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13. ABSTRACT ***(Maximum 200 Words)***

This is the second annual report on the grant "Proto-oncogene PML and tumor evasion in prostate cancer". The purpose of the grant proposal is to identify the molecular mechanisms of tumor evasion of host anti-tumor immunity. We proposed to identify the antigen presentation defects in human prostate cancer samples and to use mouse prostate cancer model (TRAMP mice) to study the immune regulation and immune tolerance in prostate cancer. In the past funding period, we have shown that thymic clonal deletion is a major mechanism for immune tolerance to tumor antigens that previously regarded as prostate specific. We provided the direct evidence that the T cell repertoire specific for tumor antigens can be shaped by negative selection in the thymus. During the process of examine antigen presentation defects in different tumor cell lines, we identified a new novel mechanism for antigen presentation gene regulation, i.e. the degradation of mRNA of an antigen presentation gene was involved in tumor evasion of immune recognition. We have also analyzed the transcription regulation of one of the antigen presentation genes and identified two new promoter regions and the essential role of the interferon response factor-binding element (IRFE) in that promoter region.

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Table of Contents

(4) Introduction

The peptides presented by class I major histocompatibility complex [MHC, in human, human leukocyte antigen (HLA)] are the primary targets on tumor cells for immune recognition by host cytotoxic T lymphocytes (CTL). Tumors can therefore avoid CTL recognition by down-regulation of cell surface MHC class I molecules. We have proposed to investigate the molecular mechanism of tumor evasion of host anti-tumor immunity. First we will identify the antigen presentation defects in prostate cancer. We will systematically examine the mRNA expression of multiple genes devoted to HLA class I antigen presentation, including HLA class I heavy chain, β_2-microglobulin (β2m), transporter associated with antigen processing genes (TAP-1, TAP-2), proteosome subunit genes (LMP-2 and LMP-7) in radical prostatectomy specimens. Next we will examine the role of proto-oncogene PML in HLA class I down regulation in prostate cancer. We will determine whether PML malfunction is responsible for HLA down regulation in prostate cancer samples. Furthermore, we will examine the immune regulation and tumor evasion mechanisms in experimental transgenic murine prostate cancer (TRAMP) models. Duing the first funding period, we have performed immunohistochemical study to show the concordant proto-oncogene PML and HLA class I antigen down-regulation in surgically removed prostate cancer lesions. We have examined the proto-oncogene PML isoform expression and antigen presentation gene expression in prostate cancer cell lines. In this funding period, we have shown that thymic clonal deletion is a major mechanism for immune tolerance to tumor antigens that previously regarded as prostate specific. We provided the direct evidence that the T cell repertoire specific for tumor antigens can be shaped by negative selection in the thymus. During the process of examine antigen presentation defects in different tumor cell lines, we identified a new novel mechanism for antigen presentation gene regulation, i.e. the degradation of mRNA of an antigen presentation gene was involved in tumor evasion of immune recognition. We have also analyzed the transcription regulation of one of the antigen presentation genes and identified two new promoter regions and the essential role of the interferon response factor-binding element (IRFE) in that promoter region.

(5) Body of Annual Report

Task 1. To identify the antigen presentation defects in prostate cancer.

- *Develop the patients' database for 308 cases of radical prostatectomy specimens we collected during the past four years. (In progress).*

  Under the support of this grant and a grant from the Cancer Research Institute, we made significant progress in establishing a new prostate cancer tissue resource in The Department of Pathology at the Ohio State University. We have a research nurse, Ms. Robinson, to coordinate the patients' consent to donate tissue for research purpose. She also started to work on the prostate cancer database. We currently collected about 80 fresh frozen samples from patients underwent radical prostatectomy. We also started to utilize the formalin fixed paraffin embedded tissue to produce Tissue Microarray. Each microarray slide contains 140 individual cylinders of tissue of 1.0 mm, which is much larger than other currently available tissue microarray, to provide more information from each tissue section.
- *Identify the prostate cancer samples that have complete loss of HLA class I expression by performing immunohistochemistry study on the archived formalin fixed paraffin embedded prostate cancer tissue samples with anti-HLA class I antibody HC10. (Completed).*

  We have completed this task. The work was presented at Experimental Biology 2001, at Orlando, Florida, on March 31 – April 4, 2001. The abstract is attached as appendix 1. The manuscript is in preparation.
- *Collect the HLA class I positive tumor tissue, HLA class I negative tumor tissue, HLA class I positive normal or hyperplastic prostatic tissue by Laser Capture Microdissection (LCM) on ethanol fixed paraffin embedded tissue sections. (In progress).*
- *Systematically examine the mRNA expression of multiple genes devoted to MHC class I antigen presentation, including MHC class I heavy chain, β_2M, TAP-1, TAP-2, LMP-2 and LMP-7 by RT-PCR and real time PCR among different groups of tissue. (In progress).*

Task 2. To examine the role of proto-oncogene PML in HLA class I down regulation in prostate cancer. .

- *Examine the PML protein expression in prostate cancer by immunohistochemistry study using monoclonal antibody against PML (PG-M3). (Completed).*

  We have completed this task. The work was presented at Experimental Biology 2001, at Orlando, Florida, on March 31 – April 4, 2001. The abstract is attached as appendix 1. The manuscript is in preparation.
- *Examine the correlation between down-regulation of HLA class I and expression of PML protein by examine the mRNA expression of PML from the tissues being collected by Laser Capture Microdissection (LCM). (In progress).*
- *Purify the genome DNA from ethanol fixed paraffin embedded tissue section and sequence the PML gene to determine if PML mutation(s) may be responsible for the defective expression of cell surface HLA class I. (In progress).*

We have systematically examined the HLA class I and PML isoform expression in various human tumor cell lines, including prostate cancer cell lines (Du145, PC3, LnCap), melanoma cell lines (SK-Mel-19, 1092, 1195), and small cell lung carcinoma cell lines (H146, H1095). We performed Northern Blot experiments to examine the mRNA level for antigen presentation genes, including MHC class I heavy chain, β_2M, TAP-1, TAP-2, LMP-2 and LMP-

7, as well as PML isoforms. During the process, we found that in a melanoma cell line SK-Mel-19, the MHC class I expression was severely depressed and transfection of PML cDNA did not restore the MHC class I expression, which was different than what we observed in a murine plasmacytoma cell line. Further analysis of this cell line revealed a novel mechanism for tumor cells down-regulate MHC class I expression. This work is summarized in Appendix 2: " A single nucleotide deletion leads to premature termination codons and degradation of TAP-1 mRNA: a potential novel mechanism for tumor evasion of host immunity". The manuscript has been submitted to *Cancer Research.*

In related question on transcriptional regulation of antigen presentation genes, we have carried out a detailed study on transcriptional regulation of TAP-2 gene. The work is summarized in Appendix 3: " Cis-elements for TAP-2 transcription: two new promoters and an essential role of the IRFE in interferon-gamma-medicated activation of the transcription initiator". The manuscript has been accepted for publication in *International Immunology*, expected in February, 2002 issue.

Task 3. To test whether overexpression of PML and upregulate MHC class I expression in vivo will improve the overall prognosis in experimental murine prostate cancer model.

- *Obtaining "TRAMP" mice from The Jackson Laboratory. (Completed).*
- *Examine the cell surface MHC class I expression in prostate cancer samples from TRAMP mice to determine whether down regulation of MHC class I heavy chain, β_2-microglobulin (β_2M), TAP-1, TAP-2, LMP-2 and LMP-7 correlates with progression of murine prostate cancer. (Superseded).*

  We have examined more than 50 samples of prostate cancer that developed in TRAMP mice. We did not find any down regulation of MHC class I expression in prostate cancer. In accordantly, we did not perform the work proposed in this subtask.
- *Breed TRAMP mice with β_2M -/- mice to determine whether ablation of MHC class I will accelerate tumor progression (Superseded).*

  Since we did not observe any difference in MHC class I expression on prostate cancer in TRAMP mice, suggesting the MHC class I expression may not play any role in tumor progression in this mouse tumor model. We did not perform the work.
- *Make the PML transgenic construct under the control of rat probasin promoter which has tissue specific expression in prostate gland (Completed)*
- *Produce the PML transgenic mice that over-express PML in prostate gland. (Completed).*
- *Breed the PML transgenic mice with TRAMP mice and determine the effect of PML on antigen presentation and tumor progression. (Completed).*

  Although we made the PML transgenic mice and PML/TRAMP double transgenic mice, we failed to identify any effect of over-expression of PML in prostate gland on MHC class I expression level, neither we find any effect on tumor incidence or progression. We considered the reason behind the failure was the prostate cancer in TRAMP mice did not down-regulate MHC class I expression.
- *Produce the costimulatory molecule B7-1 transgenic mice under the control of rat probasin promoter.(Completed).*
- *Breed the B7-1 transgenic mice with TRAMP mice, and furthermore, breed the B7-1, PML transgenic mice with TRAMP mice to examine the effects of B7 or B7 plus MHC class I expression in vivo on the incidence of spontaneous prostate cancer (Completed).*

  Although we made the B7 transgenic mice and B7/TRAMP double transgenic mice, we failed to identify any effect of over-expression of B7 costimulatory molecule in prostate gland on tumor incidence or progression. We considered the reason behind the failure

was that T cells that reactive to tumor antigen (in this model, tumor antigen is SV40 large T antigen) were deleted in thymic development as we reported below.

The transgenic adenocarcinoma of mouse prostate (TRAMP) model is transgenic for the SV40 large T antigen (Tag) under the control of the rat probasin regulatory elements. The TRAMP mice develop tumors spontaneously and orthotopically with a disease progression that closely resembles the progression of human prostate cancer. Previous studies showed that T lymphocytes from TRAMP mice are immune tolerant to SV40 Tag, while the mechanism of the tolerance is not clear. We have demonstrated that clonal deletion is a major mechanism for tolerance to tumor antigens that previously regarded as prostate specific. This work is summarized in Appendix 4: "Clonal deletion of SV40 large T antigen-specific T cells in the TRAMP mice: an important role for negative selection in shaping the repertoire of T cells specific for antigens over-expressed in solid tumor". The manuscript has been submitted to *Journal of Clinical Investigation.*

For the next funding year, we plan to continue to build the Prostate Cancer Tissue Resource with the funding from this grant and from the grant from The Cancer Research Institute. We will emphasize the work on Tissue Microarray of prostate cancer samples and to start to explore the genomic, proteomic potential of the tissue microarray and to develop new research approach for next round of Idea Development Grant proposal.

We will continue on our work in Task One and Two on Laser Captured Microdissection on different prostate tissue to identify the molecular mechanisms of tumor evasion in prostate cancer.

For the Task Three, we would like to propose to investigate the expression profile of prostate specific antigens in thymus, both in mouse and in human. Our previous work (Appendix 4) indicates that thymic clonal deletion is one of the major mechanisms for immune tolerance to antigens that previously regarded as prostate-specific. Since tumor antigens are present in a high proprtion of cancers of the same histological origin (tissue specific antigen), these antigens are the primary targets for immunotherapy. The nature of T cell repertoire to tissue antigens is of both fundamental and practical significance. Our study showed that the high affinity SV40 large T antigen specific T cells are removed from the naïve T cell pool in the TRAMP mice. Thus the strategies for immunotherapy of prostate cancer and other cancers targeting tissue or tumor antigens must take into consideration that the T cell repertoire will have been depleted the T cells of the highest affinity specific to such antigens. A new strategy may be developed under the hypothesis that preventing the thymic deletion of the tumor antigen specific T cells will have a positive impact in cancer immunotherapy.

(6) Key Research Accomplishments

- Under the support of this grant and a grant from the Cancer Research Institute, we made significant progress in establishing a new prostate cancer tissue resource in The Department of Pathology at the Ohio State University.
- We started to utilize the formalin fixed paraffin embedded tissue to produce Tissue Microarray. Each microarray slide contains 140 individual cylinders of tissue of 1.0 mm, which is much larger than other currently available tissue microarray (such as the microarray slides from NCI and U Penn), to provide more information from each tissue section.
- We have shown that thymic clonal deletion is a major mechanism for immune tolerance to tumor antigens that previously regarded as prostate specific. We provided the direct evidence that the T cell repertoire specific for tumor antigens can be shaped by negative selection in the thymus.
- We identified a new novel mechanism for antigen presentation gene regulation, i.e. the degradation of mRNA of an antigen presentation gene was involved in tumor evasion of immune recognition.
- We have analyzed the transcription regulation of one of the antigen presentation genes and identified two new promoter regions and the essential role of the interferon response factor-binding element (IRFE) in that promoter region.

(7) Reportable Outcomes:

1. Xincheng Zheng, Jian-Xin Gao, Huiming Zhang, Terrence Geiger, Yang Liu and **Pan Zheng**. 2001. Clonal deletion of SV40 large T antigen-specific T cells in the TRAMP mice: an important role for negative selection in shaping the repertoire of T cells specific for antigens over-expressed in solid tumors. *Submitted to J. Clin. Invest.*

2. Tianyu Yang, Beth McNally, Soldano Ferrone, Yang Liu and **Pan Zheng**. 2001. A single nucleotide deletion leads to premature termination codons and degradation of TAP-1 mRNA: a potential mechanism for tumor evasion of host immunity. *Submitted to Cancer Res.*

3. Yong Guo, Tianyu Yang, Xingluo Liu, Shengli Lu, Jing Wen, Joan Durbin, Yang Liu and **Pan Zheng.** 2001. Cis-elements for TAP-2 transcription: two new promoters and an essential role of the IRFE in interferon gamma mediated activation of the transcription initiator. *Int Immunol.* (In Press).

4. Huiming Zhang, Ping Wei, Jonathan Melamed, Karen Cox, Soldano Ferrone, Wendy L. Frankel, Robert R. Bahnson and **Pan Zheng**. 2001. Concordant proto-oncogene PML and HLA class I antigen down-regulation in surgically removed prostate cancer lesions: an immunohistochemical study. (In preparation).

Other publications or manuscripts in past funding period:

1. Xue-Feng Bai, Jin-Qing Liu, Xingluo Liu, Yong Guo, Karen Cox, Jin Wen, **Pan Zheng** and Yang Liu. 2000. The heat-stable antigen determines pathogenicity of self-reactive T cells in experimental autoimmune encephalomyelitis. *J. Clin. Invest.* 105: 1227-1232.

2. Yang Liu and **Pan Zheng**. 2001. Is tumor expression of the major histocompatibility complex (MHC) antigen required for T cell immune surveillance? *Arch. Immunotherapy* 49: S61-64. 2001.

3. Yannik Guilloux, Xue-Feng Bai, Xingluo Liu, **Pan Zheng** and Yang Liu. 2001. Optimal induction of effector but not memory anti-tumor cytotoxic T lymphocytes involves direct antigen presentation by the tumor cells. *Cancer Res.* 61:1107-1112.

4. Xue-Feng Bai, Jian-Xin Gao, Jinqing Liu, Jing Wen, **Pan Zheng** and Yang Liu. 2001. On the site and mode of antigen presentation for the initiation of clonal expansion of CD8 T cells specific for a natural tumor antigen. *Cancer Res.* 61: 6860-6867.

5. Xue-Feng Bai, Jonathan Bender, Jinqing Liu, Huiming Zhang, Yin Wang, Ou Li, Peishuang Du, **Pan Zheng** and Yang Liu. 2001. Local costimulation reinvigorates tumor-specific cytolytic T lymphocytes for experimental therapy in mice with large tumor burdens. *J. Immunol* 167: 3936-3943

6. Xingluo Liu, Xue-Feng Bai, Jing Wen, Jian-Xin Gao, Jinqing Liu, Ping Lu, Yin Wang, **Pan Zheng** and Yang Liu. 2001. B7H Costimulates Clonal Expansion of, and Cognate Destruction of Tumor Cells by, $CD8^+$ T Lymphocytes in vivo. *J. Exp. Med.* 194: 1339-1348.

7. Jian-Xin Gao, Huiming Zhang, Xue-Feng Bai, Jing Wen, Jinqing Liu, **Pan Zheng** and Yang Liu. 2001. Perinatal blockade of T cell central tolerance in the thymus leads to accumulation of lethal autoreactive T Cells. *Nature Medicine* (Second revision under review).

8. Xue-Feng Bai, Jinqing Liu, Kenneth F. May, Jr., Yong Guo, **Pan Zheng** and Yang Liu. 2001. B7-CTLA4 Interaction Promotes Cognate Destruction of Tumor Cells by Cytotoxic T Lymphocytes *in vivo. Blood* (Revision under review).

(8) Conclusions:

In this funding period, we have shown that thymic clonal deletion is a major mechanism for immune tolerance to tumor antigens that previously regarded as prostate specific. We provided the direct evidence that the T cell repertoire specific for tumor antigens can be shaped by negative selection in the thymus. We have identified a new novel mechanism for antigen presentation gene regulation, i.e. the degradation of mRNA of an antigen presentation gene was involved in tumor evasion of immune recognition. We have also analyzed the transcription regulation of one of the antigen presentation genes and identified two new promoter regions and the essential role of the interferon response factor-binding element (IRFE) in that promoter region. We believe that we have made important contribution to the understanding of immune regulation, especially the antigen presentation and processing, in prostate cancer and other tumors.

For the next funding year, we plan to continue to build the Prostate Cancer Tissue Resource. We will emphasize the work on Tissue Microarray of prostate cancer samples and to start to explore the genomic, proteomic potential of the tissue microarray and to develop new research approach for next round of Idea Development Grant proposal.

We will continue on our work in Task One and Two on Laser Captured Microdissection on different prostate tissue to identify the role of proto-oncogene PML and other molecular mechanisms of tumor evasion in prostate cancer.

For the Task Three, we would like to propose to investigate the expression profile of prostate specific antigens in thymus, both in mouse and in human. Our current work (Appendix 4) indicates that thymic clonal deletion is one of the major mechanisms for immune tolerance to antigens that previously regarded as prostate-specific. Since tumor antigens are present in a high proportion of cancers of the same histological origin (tissue specific antigen), these antigens are the primary targets for immunotherapy. A new strategy may be developed under the hypothesis that preventing the thymic deletion of the tumor antigen specific T cells will have a positive impact in cancer immunotherapy.

(9) References:

None.

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Experimental Biology 2001®
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March 31-April 4, 2001

ABSTRACTS 2.1-537.42
PART I

The American Physiological Society
American Society for Biochemistry and Molecular Biology
American Society for Pharmacology and Experimental Therapeutics
American Society for Investigative Pathology
American Society for Nutritional Sciences
The American Association of Immunologists
American Association of Anatomists
American Association of Veterinary Anatomists
American Association of Veterinary Immunologists
American Federation for Medical Research
AHA Council on Nutrition, Physical Activity and Metabolism
The American Society for Clinical Nutrition
American Society for Histocompatibility and Immunogenetics
American Society for Medical Laboratory Immunologists
American Society of Transplantation
Association of Latin American Physiological Societies
The Biomedical Engineering Society
Cajal Club
Clinical Immunology Society
International Society for Applied Cardiovascular Biology
International Society of Developmental and Comparative Immunology
International Society for Interferon and Cytokine Research
International Society of Neuroimmunology
International Society for NeuroImmunoModulation
The Microcirculatory Society
PsychoNeuroImmunology Research Society
Society for Cardiovascular Pathology
Society for Experimental Biology and Medicine
Society for International Nutrition Research
Society for Leukocyte Biology
Society for Mucosal Immunology
Society for Natural Immunity
Spanish Physiological Society

214.5

214.6

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Concordant Proto-oncogene PML and HLA Class I Down-regulation in Surgically Removed Prostate Cancer Lesions: An Immunohistochemical Study

Huiming Zhang[1], Jonathan Melamed[2], Karen Cox[1], Soldano Ferrone[3], Wendy L Frankel[1], Robert R Bahnson[1], Pan Zheng[1]: [1]The Ohio State University, 158 HH, 1645 Neil Avenue, Columbus, OH 43210, [2]New York University Medical Center, 550 First Avenue, New York, NY 10016, [3]Roswell Park Cancer Institute, Elm and Carlton Street, Buffalo, NY 14263

Antigen peptides presented by the class I major histocompatibility complex (MHC) molecules are primary targets on tumor cells for immune recognition by host cytotoxic T lymphocytes (CTL). As a result MHC class I down-regulation which is frequently found in malignant tumors has a negative impact on their recognition by T cells. Normal cell surface MHC class I expression requires coordinated expression of multiple genes encoding, respectively, proteosome components LMP2/7, peptide transporters TAP1/2, β_2 microglobulin(β_2M) and MHC class I heavy chain. We have previously reported that proto-oncogene product PML induces expression of TAP1, TAP2, LMP2 and LMP7 in an MHC class I negative, recurrent tumor, leading to the re-expression of cell surface MHC class I in tumors and to rejection of tumors (Nature, 396:373-376). In this study, we examined the expression of proto-oncogene product PML expression and of HLA class I antigens in 37 surgically removed prostate carcinoma lesions. Immunohistochemical staining of formalin fixed paraffin embedded sections with anti-HLA class I heavy chain monoclonal antibody (mAb) HC10 detected their down-regulation in 27 lesions (73%) with different extent (50-90% of carcinoma cells were not stained by mAb HC10). Furthermore immunohistochemical staining with anti-PML mAb PG-M3 showed that 23 of the 27 lesions (85%) with HLA class I antigen downregulation had also down-regulation of PML nuclear expression (17 cases with complete lack of reactivity to PG-M3 and 6 cases with weak reactivity to PG-M3). Morphologically, the Gleason grade 3C carcinoma that consists of well circumscribed cribriform tumor mass is the most common type that exhibits simultaneous complete loss of the HLA class I and PML expression. In summary, our results suggest that PML down-regulation is strongly associated with HLA class I down-regulation in prostate cancer.

214.9

Thymic Deletion of Specific T Cells Reactive to SV40 Large T Antigen in TRAMP Mice

Xincheng Ted Zheng[1], Jianxin Gao[1], Terrence Geiger[2], Yang Liu[1], Pan Zheng[1]: [1]The Ohio State University, 158 Hamilton Hall, 1645 Neil A, Columbus, OH 43210, [2]St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38105

The transgenic adenocarcinoma of mouse prostate (TRAMP) model is transgenic for the SV40 large T antigen (Tag) under the control of the rat probasin regulatory elements. The TRAMP mice develop tumors spontaneously and orthotopically with a disease progression that closely resembles the progression of human prostate cancer. Previous studies showed that T lymphocytes from TRAMP mice are immune tolerant to SV40 Tag, while the mechanism of the tolerance is not clear. In this study, we immunized the TRAMP mice with an immunodominant SV40 Tag epitope IV (peptide 404-411) which is presented by the class I major histocompatibility complex molecule H-2K^b, and analyzed the antigen specific T cell response by ELISPOT. We could not detect any antigen specific T cell response to Tag epitope IV in TRAMP mice, which was in consistent with the previous report. To examine whether the immune tolerance is due to thymic deletion, we crossed the TRAMP mice with TG-B mice transgenic for a rearranged T-cell receptor that recognizes Tag (peptide 559-576) presented by the class I major histocompatibility complex molecule H-2K^b. Double transgenic. TRAMP/TCR mice had thymic deletion of SV40 Tag reactive T cells when examined at 25 days after birth. The thymus size is reduced from $6.5x10^7$ thymocytes in TCR transgenic mouse to $5x10^6$ thymocytes in double transgenic mouse. The mature CD8+V$_\beta$8+ T cells from spleen are reduced from $5x10^6$ cells in TCR transgenic mouse to $1x10^5$ cells in double transgenic mouse that possibly represent the endogenously rearranged V$_\beta$8+ T cells. The thymic deletion of SV40 Tag specific T cells is identified in both male and female double transgenic TRAMP/TCR mice. We subsequently detected the message for the SV40 Tag in the thymus of the double transgenic TRAMP/TCR mice and TRAMP mice through RT-PCR and Southern blot. Our study showed that thymic deletion of T cells specific for SV40 Tag is the major mechanism for T cell tolerance in TRAMP mice.

A single nucleotide deletion leads to premature termination codons and degradation of TAP-1 mRNA: a potential novel mechanism for tumor evasion of host immunity

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Running Title: mRNA instability and TAP-1 down-regulation in tumor

Keywords: tumor immunity, antigen presentation genes, mRNA instability, post transcriptional gene regulation, melanoma.

Abstract:

Both viruses and tumors evade cytotoxic T lymphocyte (CTL)-mediated host immunity by down-regulation of antigen presentation machineries. The mechanisms underlying these abnormalities include down-regulation of transcription of antigen presentation genes and post-translational inactivation of the proteins involved in antigen presentation. So far no evidence is available to support a post-transcriptional regulation of genes involved in antigen presentation in tumors or viral infected cells. In this study, a major histocompatibility complex (MHC) class I deficient melanoma cell line SK-MEL-19 was found deficient in the expression of the transporter associated with antigen processing (TAP)-1 mRNA even after interferon-gamma (IFN-γ) stimulation, despite its active transcription of the TAP-1 gene. This abnormality is caused by a single nucleotide deletion at position +1489 of the TAP-1 gene, which results in downstream premature termination codons (PTCs) and degradation of the TAP-1 mRNA. To our knowledge, this is the first evidence that the degradation of mRNA of an antigen presentation gene is involved in HLA class I down-regulation in malignant cells, and presumably involved in tumor evasion of recognition and destruction by cytotoxic T lymphocytes.

Introduction

Recent studies demonstrate that patients with malignant melanoma often have high numbers of cytotoxic T lymphocytes (CTL) specific for melanoma-associated antigens (1-3). The co-existence of T cells and tumor cells even in the draining lymph nodes suggests that the tumors were able to evade destruction by host CTL. Accumulating evidence supports the notion that both malfunction of T cells and down-regulation of antigen presentation machinery in tumors can be responsible for tumor evasion of host immunity (4-7). In fact, a high proportion of malignant tumors, including melanoma, have severely depressed cell surface expression of class I HLA antigens, the target molecules that present tumor antigenic peptide to CTL (7). Understanding the mechanisms of the T cell malfunction or antigen presentation defects may thus provide insight for immunotherapy of melanoma and other cancers.

Optimal cell surface expression of HLA molecules requires the coordinated expression of several genes, such as transporters associated with antigen processing (TAP)-1/2, low molecular weight peptide (LMP)-2/7, tapasin, as well as HLA class I heavy chain and β_2-microglobulin (β_2M). In cases of both tumorigenesis and viral infection, expression of these genes and the function of the encoded proteins are often down-regulated (7, 8). The mechanisms for such down-regulation have been studied extensively. Theoretically, gene expression can be modulated by transcriptional, post-transcriptional, translational and post-translational mechanisms. The mechanisms that have been shown to underlie the antigen presentation abnormalities are either transcriptional suppression of antigen presentation genes, and/or functional inactivation of their gene products, either by missense mutation or by protein-protein interactions (9-12). Here we show that actively transcribed TAP-1 mRNA in the melanoma cell line SK-MEL-19 is rapidly degraded even after stimulation with IFN-γ. Cloning and sequencing

analysis have revealed that this rapid mRNA degradation is caused by a single nucleotide deletion resulting in a pre-mature termination codon (PTC). These results reveal a new potential mechanism for tumor evasion of host T cell recognition.

Materials and Methods:

***Cell lines and antibodies*:** Human melanoma cell lines 1195, 1102 and SK-MEL-19 have been cultured as described (13). The breast cancer cell line SK-BR-3 was obtained from ATCC (HTB-30, ATCC, Manassas, VA.). All the cells were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 2mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Inc., Grand Island, NY). For induction of HLA class I expression, cells were cultured in medium supplemented with recombinant human IFN-γ (1000 U/ml) (R&D Systems, Inc., Minneapolis, MN). PE-conjugated anti-HLA-A, B, C antibody (clone G46-2.6) and isotype control PE-conjugated mouse IgG1 were purchased from BD PharMingen (San Diego, CA).

***Flow cytometry*:** HLA class I expression on the cell surface of all cell lines was examined by flow cytometry as described (5). Briefly, viable cells were incubated with PE-conjugated mouse IgG1 and PE-conjugated anti-HLA-A, B, C antibody at 4°C for 2 h. After three washes with PBS containing 1% FCS, cells were fixed with 1% paraformaldehyde and examined by flow cytometry.

***Northern blot*:** The cells were treated with IFN-γ (R&D Systems, Inc.) at 1000 U/ml for 48 hrs or untreated. For cyclohexymide (CHX) (Sigma Chemical Corp., St. Louis, MO) treatment, SK-MEL-19 cells were cultured with IFN-γ at 1000 U/ml for 48 hours and then CHX were added to the cells for final concentrations of 5 μg/ml or 10 μg/ml, respectively, for up to 16 h. Total RNA was isolated using TRIzol reagent (Life Technologies, Inc.). Hybridization conditions followed the instructions of the Northern hybridization kit (Eppendorf Scientific, Inc., Westbury, NY). The cDNA probes for TAP-1, TAP-2, LMP-2, LMP-7, HLA class I heavy chain, and β2M were made from PCR products using primers listed previously (14). Human

splenocyte cDNA library from Invitrogen (Invitrogen Corp., Carlsbad, CA) was used as template for PCR reactions. All PCR products had been subcloned into pBluescript vector (Stratagene Corp., La Jolla, CA), sequenced and confirmed to be identical to published sequences. The probes were labeled with α-[^{32}P]-dCTP (NEN Life Science Inc., Boston, MA) using the DECAprime^{TM} II kit (Ambion Inc., Austin, TX).

***Generation of TAP-1cDNA constructs and stable transfection*:** Human small cell carcinoma H146 cell line (provided by Dr. N.P. Restifo, National Cancer Institute, Bethesda, MD) was incubated with IFN-γ at 1000 U/ml for 48 hours. Total RNA was isolated as above. Reverse transcription was done using the SUPERSCRIPT First-Strand cDNA Synthesis System (Life Technologies, Inc.). TAP-1 cDNA was amplified by PCR in three fragments. Primers are: hTAP.f1 5'-GCGGCCGCTTTCGATTTC GCTTTC-3', hTAP.r1 5'-TGCAGTAG CCTGGTGCTATCCG-3', hTAP.f2 5'-CTTGCCTT GTTCCGAGAGCTGA-3', hTAP.r2 5'-CTCGTTGGCAAAGCTTCGAAC-3', hTAP.f3 5'-CGGCCATGCCTACAGTTCGAAG-3', hTAP. r3: 5'-ATAAATATCAAGAACCTACAG GG-3'. The three fragments were cloned into pBS-KS vector (STRATAGENE, La Jolla, CA) at *Not*I/*Sma*I, *Sma*I/*Hind*III and *Hind*III/*Xho*I sites, respectively, and sequenced to confirm that the cDNA has a wild-type sequence. When the SK-MEL-19 cells grew to 70% confluence in the 24-well plate, 0.2 μg pcDNA3.1/Hyg(+) vector (Invitrogen Inc.) and pcDNA3.1/Hyg(+) vector with wild type TAP-1 cDNA insert were respectively transfected into each well, using 6 μl Fugene 6 transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN) according to the manual. 48 hours later, the transfected cells were re-plated onto 96-well plates and cultured in the DMEM medium and in the presence of 0.5 mg/ml hygromycin. Single cell clones were selected for further culture and analyzed for HLA class I antigen expression.

Southern blot: Genomic DNA was isolated from SK-MEL-19 cells, SK-BR-3 cells and HeLa cells. Genomic DNA (20 μg) was digested with *Afl*III (Life Technologies, Inc.) and separated in 0.8% agarose gel. The TAP-1 promoter probe was made by PCR from normal human lymphocyte genomic DNA with sense primer: 5'-TCCCGCCTCGAGCATCCCTGCAAGGCA-3' and anti-sense primer: 5'-TGCAGTAGCCTGGTGCTATCCG-3'. Probes were labeled as described above.

Generation of Luciferase-reporter constructs and assay for promoter activity: *TAP-1*promoter was amplified from SK-MEL-19 cell genomic DNA by PCR using the primers: hTAP1.Pr1 5'- GCTCTAGATGGCACTCGGACGCCGTC-3' and hLMP2.Pf1 5'-GCTCTAGACCCTGCAAGGCACCGCTC-3'. The PCR products were sub-cloned using the Zero Blunt TOPO PCR cloning kit (Invitrogen Corp.) and then cloned into pGL2-basic vector (Promega Corp., Madison WI) at *Xho*I and *Hind*III sites. All constructs were confirmed by DNA sequencing. Expression level of the firefly luciferase from the pGL2 constructs (basic, SV40, pTAP1/T and pTAP1/G) was normalized to the internal control pRL-SV40 *Renilla* luciferase level. Results were shown as the folds of increase compared with the pGL2-basic (basic). The dual luciferase assay was carried out according to the manufacturer's instructions (Promega Corp.).

Nuclear run-on assay: The assay was performed as described elsewhere (15). Briefly, nuclei were extracted from 10^7-10^8 SK-MEL-19 cells treated with 1000 U/ml of IFN-γ or without any treatment. The transcripts were labeled *in vitro* with 40 nM biotin-16-UTP (Roche Diagnostics Corp.) in the presence of 3.75 mM ATP, GTP and CTP, 25 mM Tris-HCl, 12.5 mM $MgCl_2$ and 750 mM KCl. cDNA fragments of LMP-2 and GAPDH were amplified by PCR from cloned cDNA constructs (14). TAP-1 cDNA fragment was amplified by PCR from cloned

cDNA constructs using primers hTAP1.f1 and hTAP1.r1 described above. The pcDNA3.1/Hyg(+) vector was linearized with *Hind*III. All the DNA was immobilized on nitrocellulose membrane using S&S Minifold II slot blot apparatus according to the manual (Schleicher & Schuell Inc., Keene, NH). Hybridization condition was as described before (15), and the biotin-labeled transcripts were detected using streptavidin-alkaline phophatase conjugate (Roche Diagnostics Corporation, Indianapolis, IN) and CDP-star Ready-To-Use with Nitro-Block-II reagent (Tropix Inc., Bedford, MA).

Restriction fragment length polymorphism (RFLP): Primers hTAP1CE7.f 5'-GCACCCCTCGCTGCCTACCCAGTGGTCT-3' and hTAP1E7.r 5'-TACAGGGAGTGGTAGGTTGTACCTG-3' were used to amplify the region in the TAP-1 exon 7 where the single nucleotide deletion resides from genomic DNA. The region was also amplified from cDNA using primers hTAP1E7.f and hTAP1cE7.r. PCR products were separated by gel electrophoresis and purified using QIAGEN gel extraction kit (QIAGEN Inc., Valencia, CA). The purified PCR products were incubated with *BslI* (New England Biolabs, Inc., Beverly, MA) at 55°C overnight and then separated in 5% agarose gel.

Results and Discussion

Down-regulation of TAP-1 mRNA by a post-transcriptional mechanism in melonoma cell line SK-MEL-19.

Three human melanoma cell lines, 1102, 1195 and SK-MEL-19, were examined by flow cytometry for their cell surface HLA class I expression with or without IFN-γ stimulation. A PE-conjugated anti-human HLA-A, B, C antibody was used to detect all HLA class I alleles, and a PE-conjugated mouse IgG1 was used as isotype control. As shown in Figure 1a, 1102 and 1195 cells had significant HLA class I that was further up-regulated by incubation with 1000 U/ml IFN-γ for three days. Confirming previous studies (13), we found that SK-MEL-19 cells had no cell surface HLA. Surprisingly, while other melanoma cell lines up-regulated their cell surface HLA in response to IFN-γ, very little HLA class I antigen could be found on the SK-MEL-19 even after IFN-γ-treatment.

Since optimal cell surface HLA class I expression requires the coordinated expression of multiple genes, including TAP-1/2, LMP-2/7, β_2M as well as HLA class I heavy chain, a Northern blot analysis was performed to detect the expression of these genes (Fig. 1b). In 1102 and 1195 cells, all six genes were expressed at low but detectable levels. IFN-γ-treatment drastically induced expression of all six genes. Interestingly, in the SK-MEL-19 cells, while β_2M, HLA heavy chain, LMP-2, LMP-7 and TAP-2 were present at low levels without induction, no TAP-1 mRNA was detected. After IFN-γ treatment, *β_2M*, HLA heavy chain, TAP-2, LMP-2 and LMP-7 were expressed at high levels, yet TAP-1 was still expressed at low levels.

It had been known that TAP deficient cells can express HLA class I after transfection with TAP-1 or TAP-2 gene (16-18). To test whether the lack of TAP-1 expression was responsible for the barely detectable expression of HLA class I antigen on the surface of SK-

MEL-19 cells, we transfected the cells with TAP-1 cDNA. As shown in Fig. 1c, the TAP-1 cDNA transfected SK-MEL-19 cells expressed significant levels of HLA class I antigen even prior to IFN-γ-treatment. Moreover, the TAP-1-transfectants were as responsive to IFN-γ as the other melanoma cell lines. Based on these results, it is likely that the primary defect of antigen presentation in SK-MEL-19 cells is attributable to defects in TAP-1 expression.

Since the TAP-1 expression was low at the mRNA level, the TAP-1 down-regulation may be caused by defective transcription or malfunction in the RNA metabolism. The TAP-1 expression was under the control of a bi-directional promoter, as characterized by Ting and colleagues (19). We cloned and sequenced the 593 base pair TAP-1 promoter from the SK-MEL-19 cells. In comparison to the published sequence (19), a single nucleotide G>T replacement was identified at position -446 (the first ATG of TAP-1 gene is designated as +1), which was close to the first transcription start site at -427 (19) (Fig. 2a). As the T allele resulted in a loss of restriction site *Afl*III, we did a Southern blot hybridization using *Afl*III to confirm the mismatch. As shown in Fig. 2a, while the HeLa cell line contained homozygous G alleles as described (19), both SK-MEL-19 and breast cancer cell line SK-BR-3 were homozygous for T alleles which loss the a restriction site for *Afl*III. To test if this single nucleotide replacement results in decreased promoter activity, both alleles of the TAP-1 promoter were cloned into the pGL2-basic vector that had the luciferase gene as reporter. As shown in Figure 2b, the T allele TAP-1 promoter retained 50% promoter activity of the G allele. However, given the significant variation in transient transfection and luciferase assay, it is unclear that the G>T change has significant effect on TAP-1 transcription. Importantly, both reporters were equally efficiently induced by IFN-γ treatment. Moreover, our analysis of normal human peripheral blood lymphocyte samples revealed that both alleles were present at a high frequency, and individuals

that carry either G or T alleles have equivalent cell surface HLA class I antigen expression (data not shown).

We therefore performed a nuclear run-on assay to directly evaluate the transcription of the TAP-1gene. LMP-2 transcription, which was under the control of the same bi-directional promoter, was also evaluated. As shown in Fig. 2c, TAP-1 was transcribed at high levels in SK-MEL-19 cells under basal condition, although IFN-γ appears to up-regulate TAP-1 transcription somewhat. In contrast, LMP-2 was transcribed at an undetectable level, but was induced to high levels by IFN-γ (Fig. 2c). The lack of LMP-2 transcription at basal condition may reflect the IFN-γ inducible expression pattern of this gene. These results demonstrated that lack of TAP-1 mRNA in SK-MEL-19 cells was not due to defective transcription. Taken together, the results demonstrate that a post-transcriptional defect is responsible for poor TAP-1 expression in SK-MEL-19 cells even after IFN-γ stimulation. Numerous studies have revealed defective TAP-1 expression among tumor cells (reviewed by reference 4). To our knowledge, however, this is the first example of a post-transcriptional defect of TAP-1 expression.

A single-nucleotide deletion leads to premature termination codons (PTC) and degradation of TAP-1 mRNA

A major mechanism responsible for post-transcriptional regulation of mRNA is RNA degradation, which can be prevented by cycloheximide (CHX), a protein synthesis inhibitor of mammalian cells. It is well established that the turnover of mRNA is closely linked to the translation process and blocking of translation can stabilize mRNA, especially those with short half-lives (20-22). To test if the accelerated RNA degradation is responsible for the lack of TAP-1 in SK-MEL-19 cells, we treated the SK-MEL-19 and control HeLa cells with CHX after

incubation with or without IFN-γ (1000 U/ml) for 48 hours at 37°C. At different time points after the CHX was added to the cell culture, cells were harvested and the total cellular RNA was analyzed for TAP-1 mRNA. The intensity of each band was quantified using the ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA) after exposure to a phospho-image screen. For a better comparison, TAP-1 mRNA levels were normalized to the endogenous house-keeping gene GAPDH level and the folds of increase compared with the non-CHX treated cells were then calculated. Under basal condition, the TAP-1 mRNA was up-regulated by maximal 4.8 folds in SK-MEL-19 cells. After IFN-γ induction, the TAP-1 mRNA was up-regulated by 25.7 folds. In comparison, CHX caused less significant increase of TAP-1 mRNA in both IFN-γ-treated and untreated HeLa cells. Taken together, the lack of TAP-1 mRNA, the normal transcription of TAP-1, and rescue of TAP-1 mRNA by CHX treatment indicate that the TAP-1 mRNA was rapidly degraded in the SK-MEL-19 cells. It is noteworthy that in SK-MEL-19 cells, the effect of CHX was significantly stronger when used in combination with IFN-γ. This finding cannot be fully explained by the fact the IFN-γ is a transcriptional activator for antigen presentation genes, as its effect on TAP-1 transcription is not so obvious in SK-MEL-19 cells as shown in Fig. 2c. It is likely that IFN-γ stabilized mRNA in SK-MEL-19 cells, although this possibility remains to be tested formally.

The rapid degradation of TAP-1 mRNA can be due to a genetic lesion in the TAP-1 gene. Alternatively, it is possible that the tumor cell line expressed factors that can cause TAP-1 mRNA degradation. The successful rescue of cell surface HLA class I antigen expression by wild-type TAP-1 in SK-MEL-19 cells favors the first hypothesis, since a wild type cDNA can be expressed in the tumor cell line. As the first-step to test this hypothesis, we cloned the TAP-1 cDNA from SK-MEL-19 cells that were treated with both IFN-γ and CHX. All the three clones

sequenced showed a single nucleotide deletion at position +1489 (Fig. 4a), which resides in exon 7 in TAP-1 gene. Further analysis showed that multiple downstream PTCs (the closest one is at position +1555) were present due to this nucleotide deletion (two of them were shown in Fig. 4c). To confirm that the mutation is in the TAP-1 gene, we amplified exon 7 of the TAP-1 gene from the SK-MEL-19 cells by PCR. The PCR products were digested with *Bsl*I, since this restriction enzyme recognized the deletion mutant but did not recognize the wild-type exon 7. Since complete digestion was obtained, the SK-MEL-19 cells are homozygous for this frame-shift mutation (Fig. 4b), even though the cytogenetic analysis revealed that there are 4 copies of chromosome 6 present in the SK-MEL-19 cells (data not shown). We subsequently amplified exon 7 of the TAP-1 gene from 50 normal human peripheral lymphocyte genomic DNA samples by PCR and subjected the PCR products to *Bst*I digestion. Since none of the PCR products from the 50 samples was digested by *Bst*I, it is most likely that the PTCs in SK-MEL-19 cells were resulted from a somatic mutation (data not shown).

PTCs have been shown to interfere with the metabolisms of many different mRNA in mammalian cells, leading to nonsense mediated altered RNA splicing, such as exon skipping and intron retention, or nonsense-mediated mRNA decay (20-22). Since wild type mRNA was functional in the tumor cell line, the frame-shift mutation we identified is most likely responsible for the low TAP-1 mRNA level in the SK-MEL-19 cells. Moreover, the lack of TAP-1 mRNA is probably due to nonsense-mediated mRNA decay, as CHX increased the TAP-1 mRNA level dramatically (26 folds) and no alternatively spliced TAP-1 mRNA was detected from the SK-MEL-19 cells by either RT-PCR or Northern blot. To our knowledge, this is the first report showing PTC-mediated mRNA degradation of any genes involved in antigen presentation. However, the increased turnover of HLA-C heavy chain mRNA has been suggested to contribute

to the low level of HLA-C surface expression (23). Moreover, nonsense mutation and frame-shift mutation were identified in β_2M and TAP-2 genes in tumor cells and immune deficient patients, respectively (24, 25). It is of interest to determine whether these nonsense mutations and frame-shift mutations affect mRNA level in the tumor cells by either nonsense mediated altered RNA splicing or nonsense-mediated mRNA decay.

TAP-1 protein has three major domains: transmembrane core, peptide-binding domain and nucleotide-binding domain (26). Theoretically, the frameshift mutation identified here results in a truncated protein product that lacks the nucleotide-binding domain. However, since the mRNA was degraded, it is unlikely that this protein will be produced in significant quantity. Given the importance of ATP hydrolysis in TAP-1 function, it is of interest to consider whether degradation of the mutant TAP-1 mRNA serves any useful purpose for tumor evasion of CTL recognition. A recent study from Cresswell's laboratory (27) suggested that the nucleotide binding activity of TAP-1 is less important than that of TAP-2. The truncated protein in this case may still be functional. As such, removing the TAP-1 mRNA may be essential for tumor evasion of CTL recognition.

HLA class I down-regulation has been reported in different tumor cells and may be one of the mechanisms for the tumor escape from pre-existing anti-tumor CTLs. TAP-1, as a necessary component in the HLA class I expression pathway, has also been reported to be deficient in different tumor cells (7). In primary melanoma TAP-1 down-regulation has been shown as an independent marker for poor prognosis (28). In metastatic melanoma, the frequency of TAP-1 down-regulation is as high as 83% in one report (29). It is of interest to note that our results may suggest new methods to restore antigen presentation in tumor cells. For example, in cystic fibrosis patients, one common nonsense allele, W1282X, results in unstable

mRNA. Treatment of cells harboring this allele with aminoglycosides, which promotes nonsense suppression, results in the re-expression of cystic fibrosis transmembrane conductance regulator (CFTR) on the cell surface and restoration of a cAMP-activated chloride current (30). Similar strategy might be employed in cancer immunotherapy if there is a nonsense mutation or frame shift mutation in the antigen processing genes in tumor cells that will not completely inactivate the protein.

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Figure legends.

Fig. 1. Deficiency of surface HLA class I expression in melanoma cell line SK-MEL-19 was due to the TAP-1 down-regulation. (a). HLA class I expression in three melanoma cell lines, SK-MEL-19, 1102 and 1195. Bold black lines depict the staining by PE-conjugated anti-human HLA-A,B,C antibody in untreated cells, dotted lines represent the staining by PE-conjugated mouse IgG1 as isotype control, and red lines represent anti-HLA-A,B,C antibody staining after stimulation with 1000 U/ml IFN-γ for 72 hours. (b). Expression of HLA class I heavy chain (MHC I), β_2M, TAP-1, TAP-2, LMP-2 and LMP-7 in each cell line with or without IFN-γ induction (1000 U/ml for 72 hours). Total RNA loading to each well was shown as 28s rRNA and 18s rRNA. (c). Transfection with wild type TAP-1, but not vector alone, restored the HLA class I expression in the SK-MEL-19 cells. SK-MEL-19 cells were transfected with either vector alone (top panel) or vector with TAP-1 cDNA insert (lower panel). These stable clones from each group were stimulated with or without IFN-γ and analyzed for cell surface HLA-A, B, C, as detailed in (a).

Fig. 2. Post-transcriptional mechanisms are responsible for poor accumulation of TAP-1 mRNA. (a). A single nucleotide polymorphism, adjacent to the first transcription start site (-427), was identified at -446 in the bi-directional promoter shared by TAP-1 and LMP-2 genes. The G>T change results in the loss of *Afl*III restriction site. Southern blot hybridization was performed using *Afl*III and detected by a DNA probe that encompasses the downstream region of the polymorphism site. SK-MEL-19 cells showed one 5.6 kb band that represents homozygous T allele, as did the breast cancer cell line SK-BR-3, which has significant cell surface HLA class I surface expression (data not shown). HeLa cells, in contrast, are homozygous for the G allele.

(b). Activities of T and G alleles of TAP-1 promoter (pTAP1/T and pTAP1/G, respectively) in SK-MEL-19 cells. The two allelic forms of TAP-1 promoter were cloned into the pGL2-basic vector (basic) that does not contain any promoter or enhancer but encodes the firefly luciferase. The pGL2-SV40 construct (SV40) that has both SV40 promoter and SV40 enhancer as well as the firefly luciferase reporter gene was used as the positive control. After transfection, IFN-γ was added to the cell culture at 1000 U/ml. Cells were lysed 48 hours after transfection and luciferase expression was tested using a luminometer. Data shown are representative of at least five independent experiments. (c). The TAP-1 gene was actively transcribed in SK-MEL-19 cells in the presence and absence of IFN-γ: nuclear run-on assay. Endogenous GAPDH expression was used as a positive control and the pcDNA3.1/Hyg(+) vector as a negative control. The run-on experiments have been repeated 3 times with similar results.

Fig. 3. TAP-1 mRNA level in SK-MEL-19 cells was increased by CHX. Protein synthesis inhibitor CHX was added to the SK-MEL-19 cells and HeLa cells that have normal TAP-1 and HLA class I expression. Total RNA was isolated from both cells at different time points and subjected to Northern blot hybridization to detect TAP-1 expression. The blot was exposed to a phopho-imager and the signal intensity was quantified using ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA). After normalization of TAP-1 signal to endogenous GAPDH signal in each sample, the signals in CHX treated group were compared to those that received no CHX treatment. These signals were quantitated as folds of those in untreated cells.

Fig. 4. A homozygous single nucleotide deletion was identified in the TAP-1 gene at position +1489 that resulted in premature termination codons (PTCs). (a). Sequencing chromogram.

Arrow points to the deletion site. (b). Primers hTAP1E7.f and hTAP1E7.r (arrows) were used to amplify the deletion region in TAP-1 exon 7 (E7). Arrowhead points to the position of the deletion resulting in a new *BslI* site. The PCR products of genomic DNA were purified and digested with *BslI*. Gel electrophoresis data showed that SK-MEL-19 cells were homozygous for the +1489 deletion. U, uncut; B, *BslI* digested; M, molecular weight. (c). The sequence of the deletion region was shown and the downstream PTCs (X) were underlined.

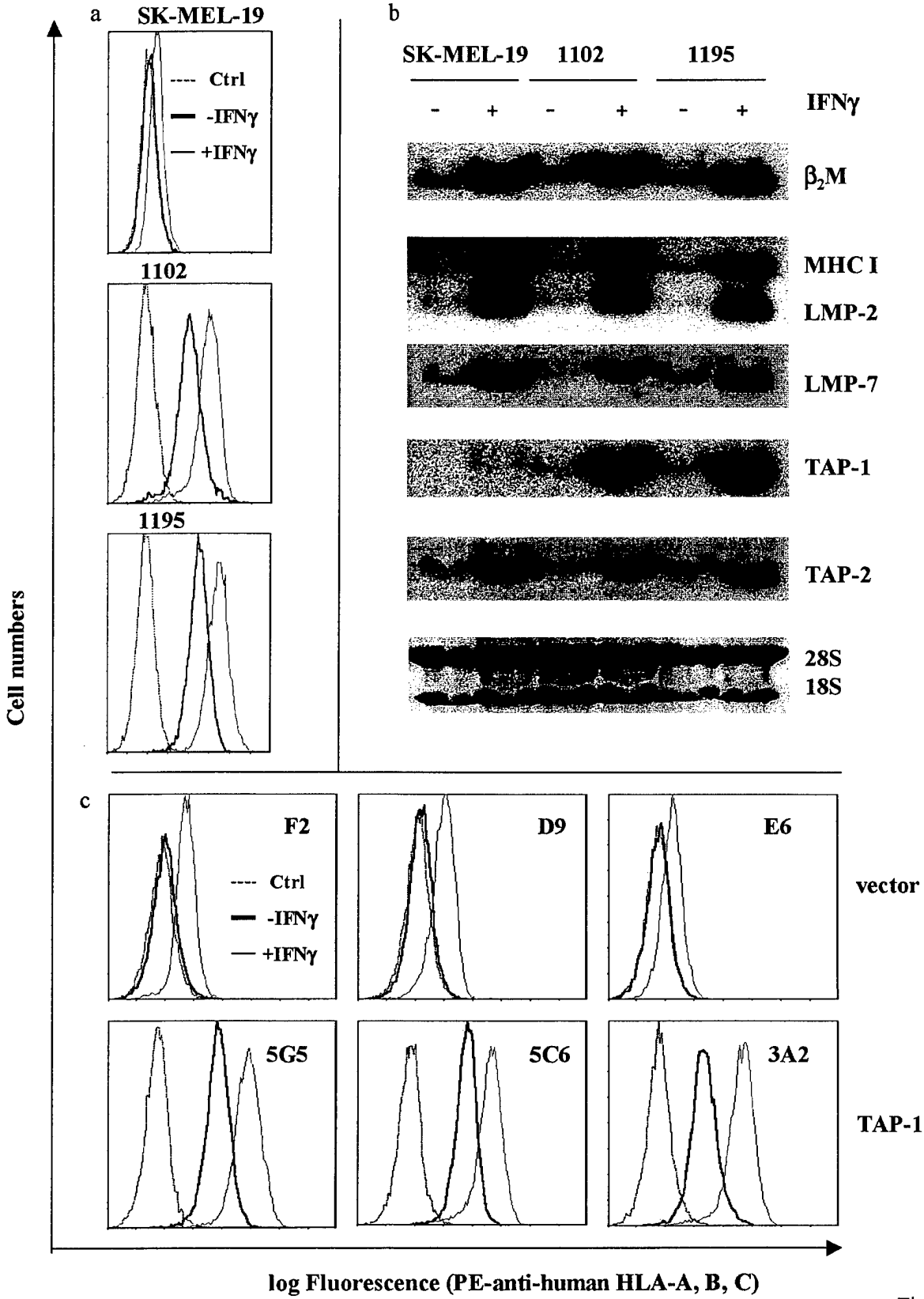

a
SK-MEL-19
---- Ctrl
-IFNγ
+IFNγ
1102
1195
Cell numbers
b
SK-MEL-19
1102
1195
IFNγ
- + - + - +
β2M
MHC I
LMP-2
LMP-7
TAP-1
TAP-2
28S
18S
c
F2
D9
E6
---- Ctrl
-IFNγ
+IFNγ
vector
5G5
5C6
3A2
TAP-1
log Fluorescence (PE-anti-human HLA-A, B, C)

Figure 1

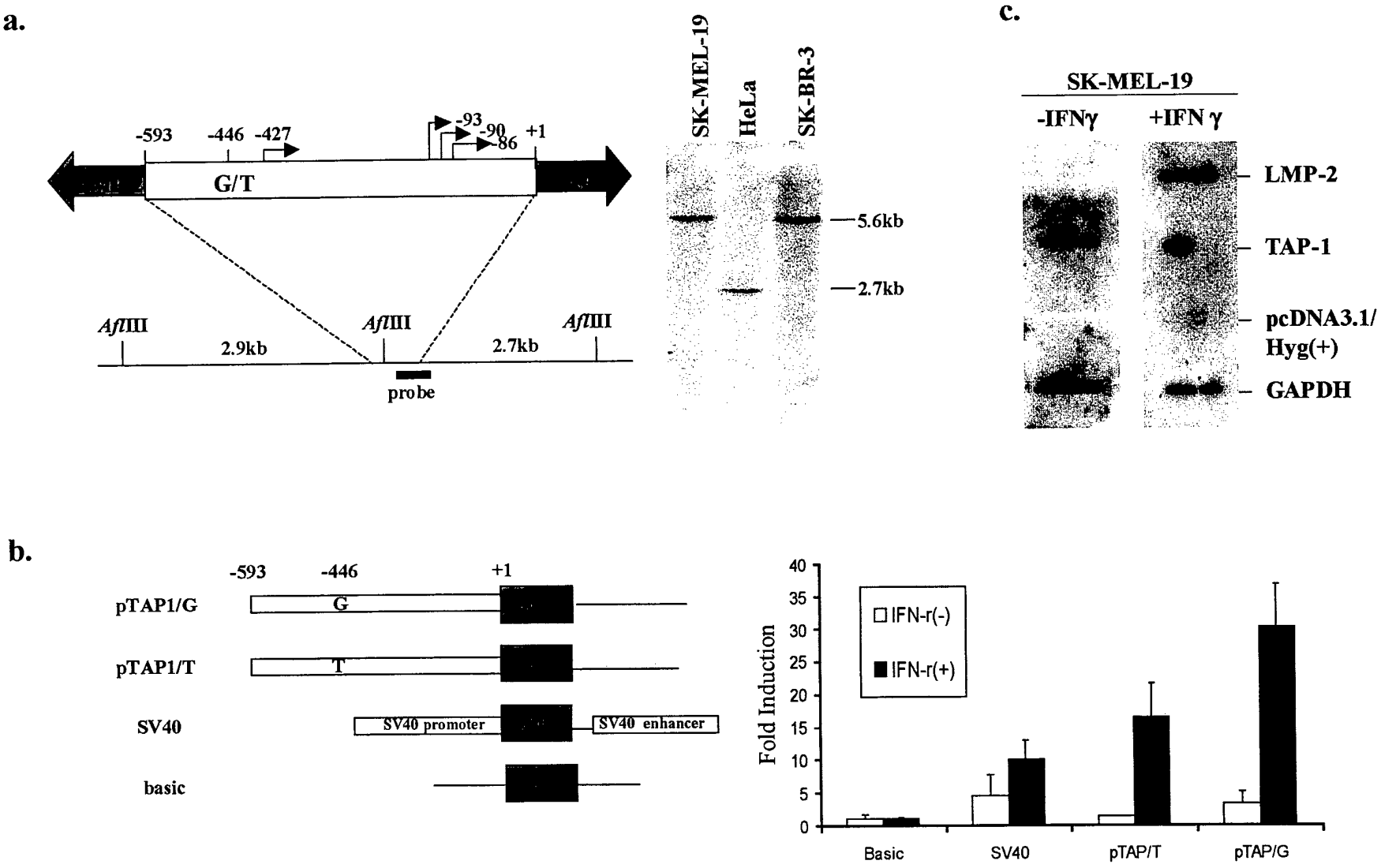

a.
-593
-446
-427
-93
-90
-86
+1
G/T
AflIII
AflIII
AflIII
2.9kb
2.7kb
probe
SK-MEL-19
HeLa
SK-BR-3
5.6kb
2.7kb
c.
SK-MEL-19
-IFNγ
+IFN γ
LMP-2
TAP-1
pcDNA3.1/
Hyg(+)
GAPDH
b.
-593
-446
+1
pTAP1/G
G
pTAP1/T
T
SV40
SV40 promoter
SV40 enhancer
basic
Fold Induction
IFN-r(-)
IFN-r(+)
40
35
30
25
20
15
10
5
0
Basic
SV40
pTAP/T
pTAP/G

Figure 2

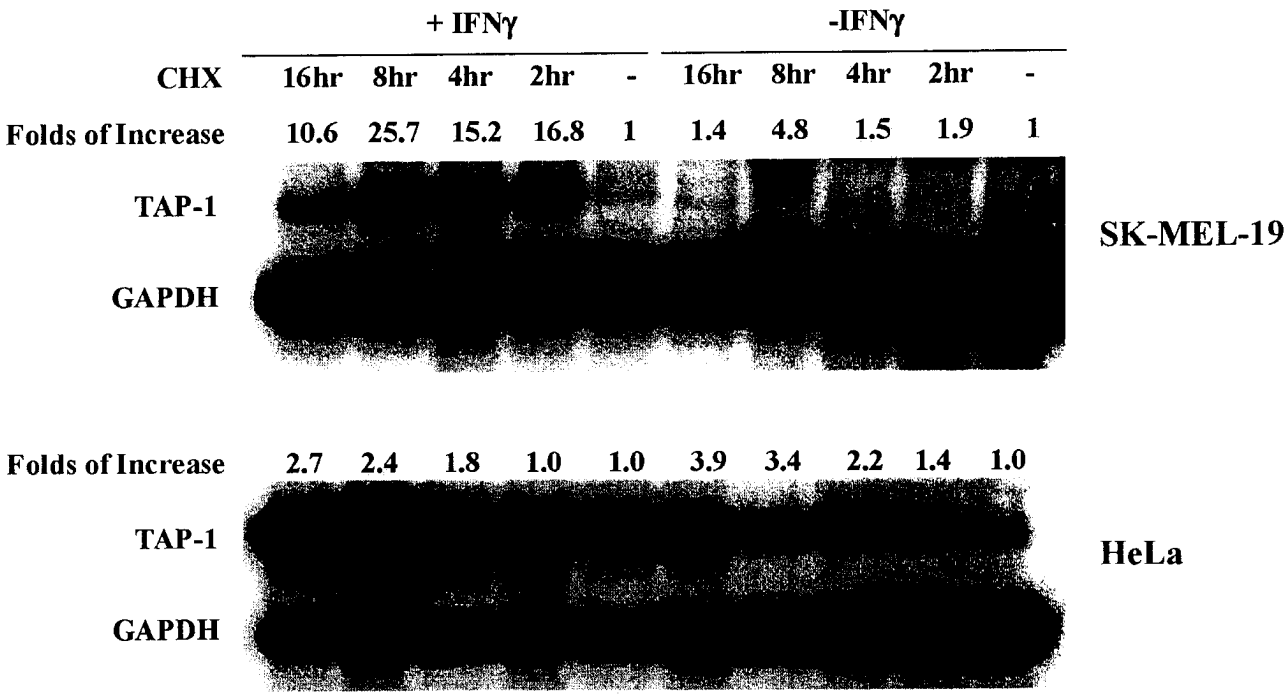
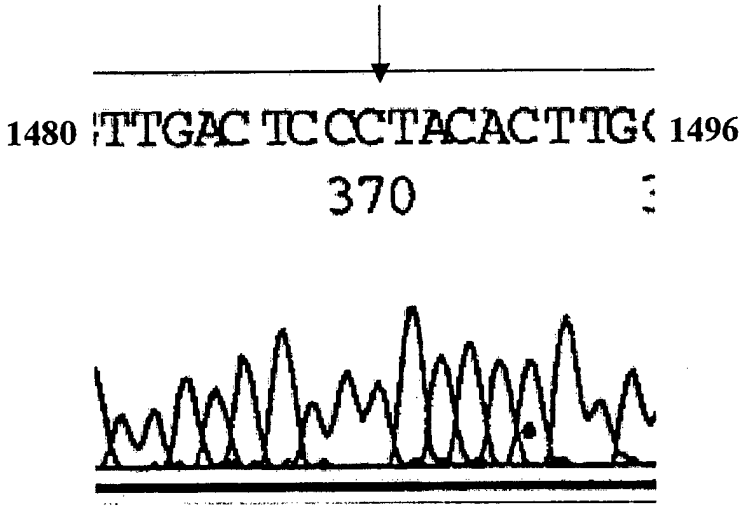
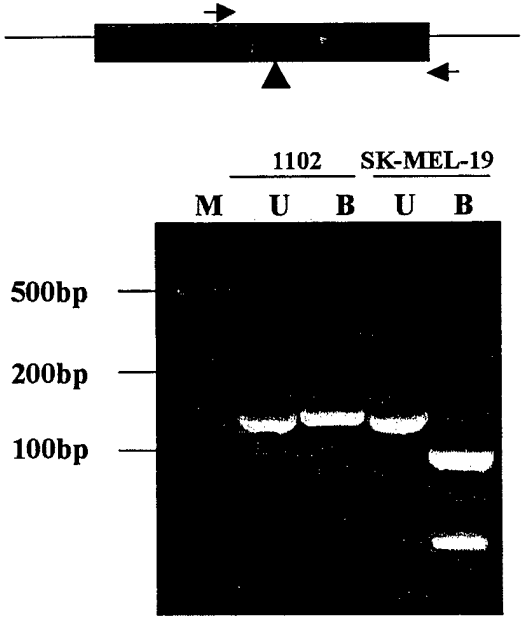

+ IFNγ
-IFNγ
CHX
16hr 8hr 4hr 2hr - 16hr 8hr 4hr 2hr -
Folds of Increase
10.6 25.7 15.2 16.8 1 1.4 4.8 1.5 1.9 1
TAP-1
GAPDH
SK-MEL-19
Folds of Increase
2.7 2.4 1.8 1.0 1.0 3.9 3.4 2.2 1.4 1.0
TAP-1
GAPDH
HeLa

Figure 3

a.



b.



c.

```
                L  T  P  Y  T  W  R  A  L  S  S  S  K  M  S  P  L  P  T  Q  T  A  Q  M  S  X  C  Y  R  G  X
Mu: 1480        TTGACTCCC-TACACTTGGAGGGCCTTGTCCAGTTCCAAGATGTCTCCTTTGCCTACCCAAACCGCCCAGATGTCTTAGTGCTACAGGGGCTGA  1572
WT: 1480        TTGACTCCCTTACACTTGGAGGGCCTTGTCCAGTTCCAAGATGTCTCCTTTGCCTACCCAAACCGCCCAGATGTCTTAGTGCTACAGGGGCTGA  1573
                L  T  P  L  H  L  E  G  L  V  Q  F  Q  D  V  S  F  A  Y  P  N  R  P  D  V  L  V  L  Q  G  L
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Figure 4

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Cis-elements for TAP-2 Transcription: Two New Promoters and An Essential Role of the IRFE in Interferon-gamma-mediated Activation of the Transcription Initiator

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Keywords: antigen presentation genes, TAP-2, transcriptional regulation, interferon γ activation.

Summary

Expression of cell surface MHC class I:peptide complex requires co-ordinated expression of multiple genes such as MHC class I heavy chain, β2 microglobulin (β2M), transporters associated with antigen-processing (TAP-1 and TAP-2) and proteosomal components LMP-2 and LMP-7. All of these genes are expressed at defined and distinct levels in normal tissues, and are inducible by interferon γ (IFN-γ). While the *cis*-elements involved in transcription of the MHC class I heavy chain, β2M, TAP-1 and LMP-2 have been analyzed extensively, those for TAP-2 and LMP-7 have not been well studied. Here we systematically analyzed the *cis*-elements for TAP-2 transcription. We found at least two independent elements that are sufficient to activate transcription of a reporter gene. One (hereby called TAP-2 P1) is located 5' to the TAP-2 exon 1, while the other (hereby called TAP-2 P2) is a transcription initiator residing in intron 1. Analysis of the 5' sequence of TAP-2 mRNA indicates that both promoters are active. Moreover, while the TAP-2 promoter region contains *cis*-elements that can mediate TAP-2 induction by IFN-γ, such as GAS and IRFE, only the IRFE is required for IFN-γ induction of TAP-2 promoter *in vitro*. The IRFE appears to work as an enhancer for the initiator (P2). Together with another promoter recently identified by others, TAP-2 therefore has 3 independent promoters that can be differentially regulated.

Introduction

MHC class I antigens are expressed constitutively in the majority of tissues, but the levels of expression differ significantly. While most leukocytes express high levels of MHC class I, other organs express these genes at much reduced levels, with almost no detectable expression in the central nervous system (1). In addition, MHC class I antigens are highly inducible by a number of cytokines, such as interferons (2), and tumor necrosis factors (3). Since MHC class I is the target for the majority of cytotoxic T cells (4), the expression level of these proteins determines the efficiency of immune surveillance by CD8 T cells. Indeed, both viruses (5,6) and tumors (7-11) can evade the immune system by down-regulating cell surface MHC class I expression.

Expression of MHC class I antigen on the cell surface requires expression of multiple genes (12-14), such as MHC class I heavy chain, β2M, peptide transporters TAP-1 and TAP-2, and immune proteosomal components LMP-2 and LMP-7, all of which are inducible by IFN-γ (15). Cell surface MHC class I expression is controlled by both transcriptional and post-translational mechanisms. Most studies on transcriptional regulation of MHC class I antigen presentation genes have focused on MHC class I heavy chain and β2M genes. More recently, the bi-directional promoter that controls both LMP-2 and TAP-1 has been studied in detail (12,16,17), but very little information is available on the promoters for LMP-7 and TAP-2. Here we have carried out a detailed deletion analysis in order to characterize the TAP-2 promoters. Our analysis has revealed two independent promoters for TAP-2 expression. Moreover, we report here that IRFE, but not the GAS element, is required for IFN-γ-mediated induction of the TAP-2 promoter, and that the IRFE acts as an enhancer for the transcription initiator.

Materials and Methods

Cells and experimental animals

A number of murine cell lines were used for the study. The NIH3T3 cell line, and the embryonic fibroblast cell lines, prepared from either wild-type (B6WT) or STAT-1(-/-) (B6STKO) C57BL/6j mice were kindly provided by Dr. David E. Levy (New York University Medical Center, New York, NY)(18) . HeLa and COS cells were obtained from ATCC. In some experiments, WT or STAT-1(-/-) C57BL/6j mice were used as sources of splenocytes.

Cloning of DNA fragment between known exons of LMP-7 and TAP-2 genes

The DNA fragment between LMP-7 and TAP-2 genes was amplified by PCR using genomic DNA from 129/sv spleen cells as the template. The LMP7.F1 was used as the forward primer and TAP2.R1 as the reverse primer. All primer sequences were listed in Table 1. PCR were carried out for 35 cycles: 94°C 1 min, 55°C 1 min, and 72°C 4 min. The 3.8 kb PCR product was cloned into pBluescript-KS vector (Stratagene, La Jolla, CA). Its identity was verified by partial sequencing from both directions. The restriction enzyme mapping of the cloned fragment was identical to the published sequence.

Construction for luciferase reporters and dual luciferase assay

A large panel of luciferase reporter constructs was made. Each construct consisted of a portion of the TAP-2 gene 5' sequence inserted 5' to the open-reading frame of the luciferase gene. TAP-2 5' gene fragments were generated by PCR using primers listed in Table 1. Since the forward primer contains a *Xho*I site, and the reverse primer has a *Hind*III site, the PCR

products were subcloned into the *Xho*I-*Hind*III sites in pGL2-Basic vector (Promega Corporation, Madison, WI).

For the IRFE enhancer activity study, P1F5/s was cut out using *Xho*I and *Hind*III and blunt-ended using T4 DNA polymerase (Life Technologies, Inc. Grand Island, NY) before being cloned into the *Sma*I site 5' to the P2 promoter in P2F9 construct. Copy number and direction of the inserts were confirmed by sequencing.

The luciferase reporter constructs were co-transfected with pRL-SV40 (Promega Corporation, Madison, WI) as a control for transfection efficiency. The promoter activity was determined using a Dual-luciferase assay kit from Promega and is expressed as fold induction, calculated according to the following formula:

Fold induction=

(sample luciferase/sample Renila luciferase)/ (basic luciferase/ basic Renila luciferase)

Data presented as means of triplicates with variations among replicates always below 20%.

Characterization of 5' sequence of the TAP-2 cDNA by PCR

cDNA libraries prepared from either RAW8.1 leukemia cell line or primary splenocytes were used as a source of TAP-2 cDNAs (19). The T7 primer was used as a forward primer, and reverse sequence corresponding to the 45-70bp down-stream of the translation initiation codon of the TAP-2 gene was used as the reverse primer (TAP2.Rev). The PCR products obtained were either sequenced directly, or were cloned into the pBluescript vector prior to sequencing.

Characterization of the 5' TAP-2 mRNA sequence by 5' RACE

Total cellular RNA was isolated from splenocytes of either wild-type or STAT-1 (-/-) C57BL/6j mice with TRIzol reagent (Life Technologies, Inc. Grand Island, NY). 5' rapid amplification of cDNA ends (5' RACE) was carried out with the 5' RACE system (Life Technologies, Inc. Grand Island, NY). Briefly, 2 μg of total RNA was used for first strand cDNA synthesis using random hexamer primers. The oligo-dC tailed cDNA was amplified by PCR using *Pfu* DNA polymerase according to the protocol from the manufacturer (Promega Corporation, Madison, WI, 35 cycles of 95°C for 1 min., 55°C for 0.5 min., 72°C for 3 min. and final extension at 72°C for 5 min.) with an abridged anchor primer (AAP, 5' RACE system, Life Technologies, Inc. Grand Island, NY) and a TAP2 specific primer (TAP2P1) corresponding to nt 454-479 of TAP2 coding sequence, (5' CCACAAGGAAGAAGAAGGCAGCTAT). A dilution of the original PCR product served as template for nested PCR using the abridged universal amplification primer (AUAP, 5' RACE system) and a second TAP2 specific primer (TAP2P2) corresponding to nt 422-437 of the TAP2 coding sequence, (5' GGCAGGTCCGGCCTGGACAGCTTCA). PCR products were separated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized to a TAP-2 cDNA probe. At the same time, larger DNA fragments (>500bp) were isolated and directly cloned into pCNTR™ shuttle vector system (Eppendorf Scientific, Inc. Westbury, NY) for the analysis. Ten TAP-2 cDNA clones were sequenced using a TAP2 specific primer (TAP2P4) corresponding to nt 325-301 of TAP2 coding sequence (5' GCCCCATAGCCAGCCAGCAGCCA). .

Results

1. Deletional analysis reveals two independent TAP-2 promoters

The murine TAP-2 cDNA available at the time of this study did not contain 5'UTR sequence upstream of the translation initiation codon (ATG). In contrast, data from GenBank showing both human (13) and rat (20) TAP-2 cDNA sequences contain a 5'UTR encoded by a distinct exon 1. As attempts to characterize the 5' UTR by primer extension and RNase protection were unsuccessful, we used RT-PCR to determine the 5' sequence of the TAP-2 gene. Two cDNA libraries cloned into the pCDM8 vector were used as templates: one was prepared from mouse splenocytes, the other from the RAW8.1 leukemia cell line. The T7 primer was used as the forward primer, and the reverse primer consisted of the nt 45-70 down-stream of the ATG initiation site (Fig. 1a and 1b). The predominant PCR products from both libraries were approximately 160 bp in length. We cloned the PCR products and sequenced three independent clones from both directions. In addition, both PCR products were subjected to bulk sequencing. The sequences of the three clones and the bulk PCR product were identical, and revealed a 119 bp sequence 5' of the ATG codon. When compared to the genomic DNA sequence, the last 8 bp preceded the ATG, and were separated from the remaining 111 bp by a 663 bp gap. Since the junction followed the GT/AG rule, it was concluded that the 663 bp insertion is an intron, which we called intron 1; while the 111 bp sequence was tentatively assigned as exon 1 (Fig. 1b and 2a). This assignment is in agreement with a recent report of Aron *et al* (21) and is consistent with the sequence of rat TAP-2 cDNA (X75305, X75306, X75307). The high GC contents in exon 1 (76/111) may explain the difficulties we encountered in primer extension and RNase protection assay.

The DNA sequence 5' of the TAP-2 open-reading frame (ORF) was not published when our analysis was initiated. Since the LMP-7 and TAP-2 genes are closely linked within the MHC class II region (Fig. 2a), we decided to isolate the intervening sequence using a pair of PCR primers based on available murine LMP-7 and TAP-2 sequences. The forward primer consisted of the 3'UTR of LMP-7 (LMP7.F1), while the reverse primer sequence corresponded to the 5' coding exon of TAP-2 (TAP2.R1). Using these primers, a 3.8 kb PCR fragment was amplified from 129/sv mouse spleen DNA and cloned into pBS-SK vector. Partial DNA sequence of approximately 600 bps from either terminus revealed that the 5' sequence of the 3.8 kb fragment was identical to the LMP-7 3' UTR, while the 3' sequence was identical to 5' portion of TAP-2. While this work was in progress, 138kb of sequence from the murine MHC class II region was submitted to GenBank by Hood and colleagues (AF027865). The partial sequence we obtained was identical to the region between LMP-7 and TAP-2. Further restriction mapping indicated that the DNA fragment we had cloned was identical to the published sequence (data not shown).

As a first step towards characterizing the promoter region that controls TAP-2 expression, we compared the promoter activity of a 1.7 kb 3' fragment (P1F1) with that of the full length 3.8 kb fragment (P1P2). The two fragments were cloned into the pGL2 luciferase reporter vector, and transiently transfected into NIH3T3 or embryonic fibroblast cell lines prepared from either wild-type or STAT-1(-/-) C57BL/6j mice. After 48 hours, the cell lysates were analyzed by dual-luciferase assay. As shown in Fig. 2 b-d, both P1F1 and P1P2 fragments had strong and comparable promoter activity. We therefore restricted further analysis on the 1.7 Kb P1F1 fragment.

As illustrated in Fig. 2a, the P1F1 fragment contained approximately 1 kb of sequence 5' to exon 1, all of exon 1 and intron 1, as well as part of the exon 2 sequence. Analysis of P1F1 revealed multiple potential transcription factor binding sites, as depicted in Fig. 3a. These *cis*-elements were scattered in the region 5' of exon 1, and in intron 1. To determine the potential contribution of these *cis*-elements, we generated a series of promoter deletion mutants and inserted these fragments into the pGL-2 luciferase vector (Fig. 3b). Upon transfection of these constructs into both murine (NIH3T3) and human (HeLa) cell lines, we consistently observed undiminished promoter activity when all but 70 bp of the sequence 5' of the exon 1 was deleted (Fig. 3c). Further deletion in the 5' region, as in the case of P1F5 resulted in a 3-fold reduction of the promoter activity. Moreover, removal of an additional 32 bp 5' of exon 1 (P2Sac) reduced promoter activity by another 5-10 fold. Noted here that P2Sac fragment that contains exon 1 and intron 1 still maintains significant promoter activity. Nonetheless, the 70 bp fragment 5' of exon 1 plays an important role in transcriptional activation of the TAP-2 promoter.

To test whether the 70 bp and the 32 bp fragments are sufficient to activate transcription, we inserted the 70 bp (P1F4/s) or the 32 bp element (P1F5/s) into the luciferase vector (Fig. 4a), and compared their activity to that of the SV40 promoter. As shown in Fig. 4b, both had significant promoter activity but the 30 bp (P1F5/s) fragment was much less potent. These fragments had neither TATA box nor SP1 sequence, but were remarkably G/C rich.

Nevertheless, the 70 bp fragment (P1F4/s) had a substantially lower promoter activity than the entire P1F4 fragment, and the significant promoter activity observed in the P2Sac fragment (Fig. 3b), suggested the existence of additional *cis*-elements within the intron 1. We therefore generated another series of reporter constructs in which partial intron 1 sequences were inserted 5' to the luciferase gene (Fig. 5a). Since two potential CRE sites (in reverse orientations)

could be recognized in the intron 1, we designed our deletion fragments to test the function of these known elements. In three cell lines, we observed that all 4 fragments tested had measurable promoter activity (Fig. 5b-d). Removal of either one (P2F4 in Fig. 5b) or two (P2F5 in Fig. 5c and 5d) of the CRE sites reduced the promoter activity by 2-5 fold, suggesting that these CRE sites have positive role in TAP-2 activation. Interestingly, promoter activity was restored when an additional 32 bp fragment was deleted (P2F8). It is therefore possible that this fragment may have a negative regulatory role that can be neutralized by the CRE sites. Since the minimal 38 bp fragment (P2F9) had activity virtually identical to that of the longest fragment (P2F1), all the promoter activity of the intron 1 must be therefore resided within this 38 bp fragment (P2F9). Consistent with this conclusion, the 38 bp fragment contains a typical initiator (Inr) sequence TCA(+1)TTTC.

2. 5' Race revealed that the transcription initiator is functional *in vivo*.

The deletional analysis described above revealed that TAP-2 gene has two promoters: one resides just 5' of exon 1.and the other is potentially a transcription initiator (Inr) residing within intron 1. A unique feature of the Inr is that transcription starts at the +1 site of the CTA(+1)TTTC, which serves as a useful fingerprint of the Inr utilization (22,23). Since utilization of the two promoters should result in distinct products, we set out to characterize the 5' UTR of the TAP-2 transcripts. As discussed, we were unable to indentify the 5' transcription start sites by primer extension and therefore took the 5'RACE approach. We made a reverse primer using TAP-2 exon 2 sequence, and amplified the 5' sequence using RNA isolated from either wild-type or STAT-1(-/-) splenocytes. The PCR products were cloned into the pBlusescript vector, and the TAP-2 clones obtained were individually sequenced. These

sequences were aligned with the genomic DNA sequence (Fig. 6a), and the results are summarized in Fig. 6b. In addition to the 5' sequence shown in Fig. 1, which we called TAP-2-1, the 10 clones sequenced fell into 3 groups. Group 1 (TAP-2-2) has all but 28 bp of exon 1 sequence (1/10, from the STAT-1(-/-) spleen cells). The close proximity and the spacing between the 5' of the TAP-2 cDNA (TAP-2-1 and TAP-2-2) and that of the promoter 1 identified in this study strongly suggest that the promoter 1 (TAP-2 P1) is responsible for transcription of this product.

Half of the clones sequenced belong to group 2 (TAP-2-3), which started precisely at the +1 site of the Inr (Fig. 6a). This precise initiation strongly suggested that they are the products of transcription using Inr as the promoter. Group 3 clones (TAP-2-4) have a 5' terminus 9 bp down-stream of the putative translation initiation ATG codon. Because a second in-frame ATG start codon was present at position 49 after the first, it is possible that TAP-2-4 mRNA can still encode a truncated TAP-2 protein. Nevertheless, it is unclear whether this last group of cDNA reflects real mRNA product *in vivo* and if so, whether this truncated TAP-2 protein can be functional.

In order to measure the relative abundance of the three groups of the TAP-2 cDNA, the 5'RACE products were separated by agarose gel electrophoresis and then analyzed by Southern blot using TAP-2 cDNA as the probe. Inserts from all three groups of cDNA clones were isolated and used as markers. As shown in Fig. 6c, 4 major species of the 5' RACE products were detected by Southern blot. The molecular weights of these bands were consistent with the 4 transcription start sites we have predicted. It is therefore likely that the 5' termini of the major TAP-2 mRNA species have now been identified. Moreover, since all species were present in the

RACE products from both WT and STAT-1(-/-) spleens, both promoters must have been functionally independent of STAT-1.

3. IRFE as an essential IFN-γ responsive element that acts as an enhancer for Inr.

An important feature of genes involved in antigen presentation is their induction by the interferons, especially the IFN-γ. The TAP-2 promoter region contains an IFN-γ activation site (GAS) and an interferon response factor binding element (IRFE). To determine whether any of these *cis*-elements are required for IFN-γ-mediated induction of TAP-2, we carried out a systematic deletion analysis. The deletion mutants were cloned into the luciferase reporter constructs, as illustrated in Fig. 7a, and then used to transfect either wild-type or STAT-1(-/-) embryonic fibroblasts. Transfected cells were left untreated or treated with 1000 U/ml of IFN-γ, and lysates were tested for luciferase activity at 48 hours. The ratios of luciferase activity in IFN-treated over untreated cultures were presented in Fig. 7b. The data showed that deletion of all but 32 bp 5' of exon 1 had no effect on IFN-γ induced TAP-2 promoter activity. Since the deletion of GAS had no effect on IFN-γ responsiveness, the GAS was not necessary for induction by IFN-γ. In contrast, IFN-γ induction was eliminated after a 32 bp sequence was removed. Regardless of the constructs used, IFN-γ function depends on the STAT-1 gene, as the STAT-1(-/-) fibroblasts showed no induction of TAP-2 activity by IFN-γ.

Since the essential 32 bp fragment contains an IRFE, it is most likely that this element is required for IFN-γ responsiveness. To confirm this, we generated a mutant that contained all TAP-2 5' sequence except the IRFE (Fig. 8a). As shown in Fig. 8b, the response to IFN-γ was completely eliminated by deletion of the IRFE. Thus IRFE is required for IFN-γ-response of the TAP-2 promoter. Again, despite the fact that promoter activity was unaffected by deletion of

GAS, IFN-γ function was strictly dependent on the STAT-1 gene as TAP-2 was not induced by IFN-γ in fibroblasts derived from the STAT-1(-/-) mice.

To test if the P1, which contains the IRFE, is responsive to IFN-γ, we compared P1F1, P1F4/s and P1F5/s activity in cells with and without IFN-γ stimulation. As shown in Fig. 9a, while P1F1 promoter was highly responsive to IFN-γ-stimulation, the response of P1F4/s and P1F5/s to IFN-γ was not significant. This result suggests that the IFN-γ cannot stimulate IRFE to enhance P1 activity. An alternative hypothesis is that IRFE works in concert with the Inr. To evaluate this possibility, we linked, in different copy numbers and orientations, the P1F5/s fragment, which contains the IRFE, to the P2F9 fragment, which contains the Inr. Since the P2F9 by itself was not responsive to IFN-γ (data not shown), we defined the activity of P2F9 as 1 to better illustrate the function of IRFE. As shown in Fig. 9b, a single copy of P1F5/s significantly increased the P2F9 activity and additional copies of the P1F5/s increased the promoter activity. The reverse orientation appears somewhat more active. These results suggest that IRFE can act as an enhancer for the Inr residing in the intron 1.

Discussion

The TAP-2 gene encodes an essential subunit for the transporter associated with antigen processing. While it is clear that transcriptional regulation of TAP-2 is an important aspect of antigen presentation, very little information is available on the gene structure and regulation of TAP-2 transcription. Our results presented here, and a recent publication by Arons et al. (21), provide the much-needed initial characterization. Taken together, the two studies reveal three distinct promoters responsible for expression of TAP-2 mRNA, two of which are responsive to the IRFE-mediated induction by IFN-γ.

The first promoter, P1, resides immediately upstream of exon 1. Deletion analysis revealed that essentially all of the promoter activity is contained in a 70-bp fragment 5' of exon 1. A lower, but significant promoter activity can be detected in an even smaller 32 bp fragment containing the IRFE sequence. Interestingly, while these short fragments have promoter activity, they contain neither a TATA box nor an Sp1 consensus sequence. The high G/C content of this region may allow binding of RNA polymerase to initiate transcription. The existence of this promoter is supported by Arons et al. (21), who reported a significant promoter activity of a 95-bp fragment encompassing both the 32 bp and 70 bp fragments. The second promoter, P2, reside 38 bp 5' of the translation start codon. Sequence analysis of this region indicates that a transcription initiator (Inr) is located within the region. A signature of Inr function is that transcription starts at the +1 position of the Inr (22,23). Analysis of the 5' sequence of TAP-2 transcripts identified using 5'RACE indicates that the Inr is indeed employed as a second promoter. However, Arons et al (21) were not able to observe any TAP-2 transcripts initiated in the intron 1 by RNase protection assay. This is most likely due to technical difficulties associated with the high G/C content of this region, as the majority of the 5' initiation sites were not

identified by this method (21). The third promoter, identified by Arons et al. (21) (hereby called TAP-2 P3), encompasses 111-bp of exon 1. This region contains two MED1 (multiple starting site down stream element) sequences which may explain the multiple initiating sites identified by Arons et al. (21). It is unclear whether the difference in our results is due to technical difficulties or reflects the use of different cell lines in our respective studies.

It is worth noting that while all three promoters can function independently, in physiological context they are most likely to function in concert. This is underscored by the requirement of the first intron for optimal functioning of the first promoter, P1. The precise sequence in the intron 1 that enhances the P1 activity remains to be characterized. Sequence analysis revealed that the DNA that encodes intron 1 contains two CRE elements. As a group, these *cis*-elements do not enhance the function of the Inr. It remains to be tested if they are responsible for increasing the efficacy of the promoters 1 and 3.

Although the initiator alone has strong promoter activity, this activity appears to be negatively regulated by the additional sequence in the 5'. Since the initiator was preceded by two CRE elements that have been implicated in suppression of MHC class I heavy chain transcription (24), we tested the effect of deleting CRE on the activity of the initiator. Our results demonstrated that deletion of the two CRE elements reduces the initiator activity, thus the CRE elements are not negative regulators for the TAP-2 initiator.

Our data and that of Arons et al. (21) have shown that IRFE is required for IFN-γ-mediated induction of TAP-2 promoters 2 and 3, respectively. In addition, the IRFE is required for optimal constitutive activity of promoter 3 (21), as is generally true for MED1-promoters (25). However, linking the IRFE to the Inr did not appreciably increase the Inr's constitutive activity. Since the IRFE is part of the P1, it obviously plays a role in its constitutive activity.

Most, if not all, transcriptional activation by interferon γ is mediated by the Jak-Stat pathway (18,26,27). The IFN-γ signaling process is initiated by binding of dimeric IFN-γ to the ligand binding IFN-γ receptor (IFNGR) α subunit chain. This leads to receptor dimerization, which is followed by activation of the Janus protein tyrosine kinases, Jak-1 and Jak-2, associated with the IFNGR subunits. Subsequently, this leads to the phosphorylation of latent cytoplasmic transcription factor Stat-1 and the translocation of activated dimeric Stat-1 to the nucleus. The GAS response element TTCC(C or G)GGAA present within responsive promoters is bound directly by the Stat-1 dimer, thereby leading to transcriptional activation. Several GAS-like elements that possess the palindromic core sequence TTN_5AA are also bound by Stat-1 (26-29). The GAS element in TAP-2 P1 region has the sequence of TTCCTCAAA, which more likely to be classified as GAS-like element. Our study revealed that in the STAT-1(-/-) fibroblast the TAP-2 promoter is completely resistant to IFN-γ-mediated induction. However, the critical involvement of the IRFE, but not the GAS element, in IFN-γ mediated activation of TAP-2 promoter argues for a different mechanism. Several lines of evidence support this. First, Arons et al (21) showed that the IRF-1, but not IRF-2 and ICSBP (IFN consensus sequence binding protein), is sufficient to induce the TAP-2 promoter. Second, Stat-1 is required for optimal expression of IRF-1 (18,30). In this regard, it is most likely that activation of TAP-2 promoter requires both Stat-1 and IRF-1, much as what has been suggested for LMP-2 (31). Recent studies revealed an alternative pathway in which interferon activated Stat-1 complexed with IRF-9 (p48) can activate transcription through interaction with the ISRE (32-34). The function of IRF-9 in the binding of TAP-2 promoter is not clear, as our preliminary studies indicated that anti-p48 did not super-shift the IRFE-protein complex from IFN-γ stimulated cells (data not shown).

In summary, our analysis and that of Arons et al.(21) indicate that, despite the presence of numerous *cis*-elements, relatively few are involved in both constitutive and IFN-γ inducible expression of the TAP-2 gene. The constitutive expression is controlled by three promoters, a 70-bp fragment 5' to the exon 1, the MED1 sequence within exon 1, and an initiator located 32 bp 5' of the translation start codon. Identification of these *cis*-elements will facilitate characterization of the transactivating element that controls the TAP-2 gene expression. Moreover, since the promoter activity dissected in our study is based on analysis of five different cell lines from three different species, murine, monkey and human, it is most likely that the elements identified may function in a number of contexts. The existence of distinct promoter and transcription initiation sites allows independent mechanisms for expression of TAP-2. This apparent redundancy will likely make it more difficult to inactivate transcription by virus and tumors, while providing ample opportunities for interplay of multiple pathophysiological factors.

Abbreviations:

5'RACE: rapid amplification of 5' cDNA end;

CRE: cAMP response element;

GAS: gamma activation site;

IFN-γ: interferon gamma;

IRF: interferon regulatory factor;

IRFE: interferon response factor binding element;

ISRE: interferon stimulated response element;

LMP: low molecular weight polypeptide;

MED1: multiple start site element down-stream;

TAP-2: transporter associated with antigen processing-2.

Footnote:

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Table 1. Primer List.

1. Primers for identifying transcription start sites from cDNA library.

T7. F	5′ GCC AGT AAA TTG TAA TAC GAC TCA CTA TAG 3′
TAP2.Rev	5′ ATA AAG CTT GTA GCA ACC CAA GTA AAG CCA TGT CC 3′

2. Primers to clone DNA fragment between LMP-7 and TAP-2.

LMP7.F1	5′ GGC CCC CGC ACT CAC AGA TAC CTT CTA AG 3′
TAP2.R1	5′ ATA AAG CTT GTA GCA ACC CAA GTA AAG CCA TGT CC 3′

3. Primers for pGL2 constructs in deletion analysis.

pTAP2.R2-H3	5′ GGA AAG CTT GGT GGG TTC TGC GGA AAG 3′
P1F1.XHO	5′ AAC CTC GAG TCA TTT CTC CTT GTA 3′
P1F2.XHO	5′ GAC CCT CGA GAG TTT TGC TAG 3′
P1F3.XHO	5′ CAT GCT CGA GGT CGG AGA AAT 3′
P1F4.XHO	5′ CCC CTC GAG GCG CCA TCT GCT G 3′
P1F5.XHO	5′ CCC CTC GAG CGC GAC CAC GGC AGT 3′
P2Sac.XHO	5′ CCC CTC GAG CGC GGG ACC CAG GCG 3′
P2F1.XHO	5′ AAC CTC GAG AAG TTG CAG GAG TGC 3′
P2F2.XHO	5′ AAC CTC GAG GCT ACC GAA CCC ACC 3′
P2F3.XHO	5′ AAC CTC GAG ATG GAT GTG GTG GCA 3′
P2F4.XHO	5′ AAC CTC GAG AAT AAT GCA GCC TTC 3′
P2F5.XHO	5′ CCC CTC GAG AGA CTT ATC TAG 3′
P2F8.XHO	5′ CCC CTC GAG GCT CGT AAC CAG 3′
P2F9.XHO	5′ CCC CTC GAG ACC TCA TTT CTC C 3′
P1R2.H3	5′ AAA AGC TTC TTC TCA AAC TGG A 3′

4. Primers for 5′RACE.

TAP2.P1	5′ CCA CAA GGA AGA AGA AGG CAG CTA T 3′
TAP2.P2	5′ GGC AGG TCC GGC CTG GAC AGC TTC A 3′
TAP2.P4	5′ GCC CCA TAG CCA GCC AGC AGC CA 3′

Figure Legends

Fig. 1 Searching for TAP-2 exon 1 by RT-PCR. a. Flow-chart of PCR and cloning of TAP-2 5' fragment. Two pCDM8 (Invitrogen Corp., Carlsbad, CA) based cDNA libraries, one from the murine splenocytes, and the other from the murine leukemia cell line RAW8.1, were used as templates. The T7.F forward primer and the TAP2.Rev reverse primer, which was based on sequence of TAP-2 cDNA 45-70 bp down-stream of the translation initiation site, were used to amplify TAP-2 cDNA. The PCR products were cloned into the pBluescript vector. b. Sequence of TAP-2 cDNA and alignment with genomic DNA. The inserts of three clones were sequenced and found to be identical. The sequence is also confirmed by bulk sequence of the PCR products. The first 111 bp were separated from the remaining cDNA fragment by a 663 bp fragment. The DNA encoding the 111 bp is hereby assigned as exon 1.

Fig. 2. Cloning the TAP-2 promoter region. a. Diagram of the 5' region of the TAP-2 gene in the context of mouse MHC region in the chromosome 17. The bold arrows indicate the direction of transcription, while the small arrows indicate the primers used to amplify the 5' region of TAP-2. b-d. A 1.7 kb fragment of the TAP-2 5' sequence has optimal promoter activity in three different cells lines. The full length 3.8 kb DNA fragment between LMP-7 and TAP-2 (P1P2) and the 1.7 kb DNA fragment (P1F1) were cloned into the pGL2-Basic luciferase vector and transiently transfected into NIH3T3 cells (b), embryonic fibroblast cell lines prepared from either wild-type (B6WT)(c), or STAT-1(-/-) (B6STKO) (d) C57BL/6 mice. The luciferase activity in cell lysates was analyzed by dual-luciferase assay after 48 hours. The pGL2-Basic (Basic) and pGL2-SV40 (SV40) from Promega were used as control.

Fig. 3. Identification of the first promoter sequence 5' of exon 1. a. Known *cis*-elements in the 1.7 kb DNA fragment 5' to TAP-2 (P1F1). Numbering of the sequence is relative to the ATG codon of TAP-2 (thick bent arrow). The sequences of potential *cis*-elements are boxed, the sequences of exon 1 and part of exon 2 are underlined, and three transcription starting sites (TSS) are indicated by bent arrows. b. Diagram of the deletion mutants. c. Deletion analysis revealed the critical function of a 70 bp fragment, 5' of exon 1, for the promoter activity of the 1.7 kb fragment. Constructs with deletions upstream of exon 1 were transfected to either NIH3T3 or HeLa cells, and the promoter activity was determined by the dual-luciferase assay.

Fig. 4. The 70 bp and 32 bp fragments 5' of exon 1 have significant promoter activity. a. Diagram of the *cis*-elements in the P1 region, and the constructs used. b. Promoter activity. Note detectable promoter activity of the IRFE sequence (P1F5/s) and the significant enhancement of the promoter activity due to additional 40 bp fragment upstream (P1F4/s).

Fig. 5. Characterization of TAP-2 promoter 2. a. Diagram of the *cis*-elements in P2 region, and the constructs used. b-d, The constructs were transfected into HeLa (b), NIH 3T3 (c), and COS cells (d) to test their promoter activity 48 hours after transfection.

Fig. 6. Identification of the 5' termini of TAP-2 RNA by 5' RACE: evidence for utilization of the initiator in spleen cells. a. Alignment of four groups of TAP-2 transcripts with TAP-2 genomic DNA sequence. b. Summary of the clones isolated by two different strategies. c. Relative abundance of the four species of TAP-2 RNA. The 5' RACE products were separated

by 1.5% agarose gel electropheresis, and then transferred to nylon membrane. The membrane was probed with TAP-2 cDNA.

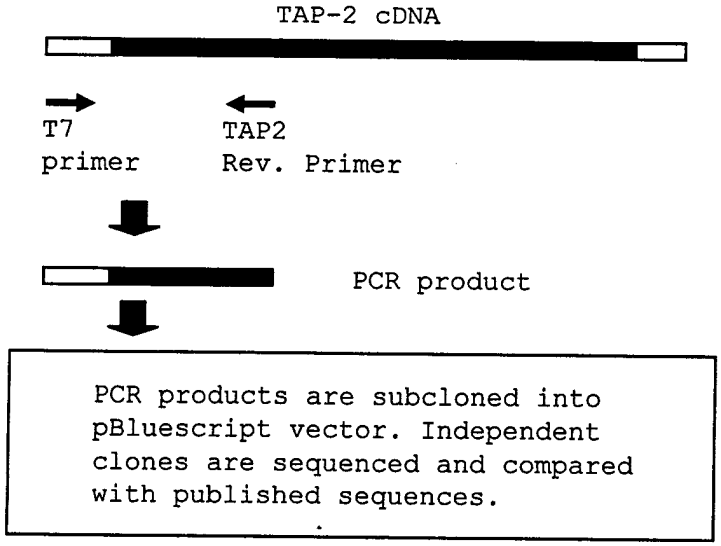
Fig. 7. IRFE, but not a GAS element, is involved in IFN-γ-mediated induction of the TAP-2 promoter activity. a. Diagram of the *cis*-elements in P1 and P2 regions, and the constructs used. b. The constructs were transfected into the embryonic fibroblast cell lines prepared from either wild-type (B6WT, filled bars) or STAT-1(-/-) (B6STKO, open bars) C57BL/6j mice. After transfection, the cells were incubated with medium, or medium containing 1000 U/ml of IFN-γ for 48 hours and the promoter activity was determined. Data presented are the ratio of fold induction of promoter activity from IFN-γ-treated culture over that from the untreated culture.

Fig. 8. The IRFE element is essential for IFN-γ-mediated induction of the TAP-2 promoter activity. a. Diagram of *cis*-elements in P1 and P2 regions and constructs P1F1 and P1F1 with deletion of the IRFE element (dIRFE). b. and c. The constructs were transfected into the embryonic fibroblast cell lines prepared from either wild-type (B6WT, b) or STAT-1(-/-) (B6STKO, c) C57BL/6j mice. After transfection, the cells were incubated with medium (open bars), or medium containing 1000 U/ml of IFN-γ (filled bars) for 48 hours and the promoter activity was determined. Data shown were means and SD of luciferase activity expressed as fold induction.

Fig. 9. The IRFE is an IFN-γ-activated enhancer for the transcriptional initiator. a. Promoter 1 is not responsive to IFN-γ-stimulation. The constructs P1F1, P1F4/s, P1F5/s (as in Fig. 4a) were transfected into the embryonic fibroblast cell lines prepared from either wild-type (B6WT) or

STAT-1(-/-) (B6STKO) C57BL/6j mice. After transfection, the cells were incubated with medium (-IFN-γ), or medium containing 1000 U/ml of IFN-γ (+IFN-γ) for 48 hours and the promoter activity was determined. Data shown were means and SD of luciferase activity expressed as fold induction. b. The IRFE enhances the initiator activity upon IFN-γ stimulation. The 32 bp P1F5/s, which contains the IRFE, was inserted in the 5' of the P2F9, which contains the Inr, in different copy numbers and orientation as indicated. The constructs were transfected and assayed as described in a. The data were normalized over the activity of P2F9 to illustrate the function of IRFE. Data shown were representative of 3-5 independent experiments.

a. Identification of TSS by PCR from two cDNA libraries



b. TAP-2 cDNA 5'end PCR sequence

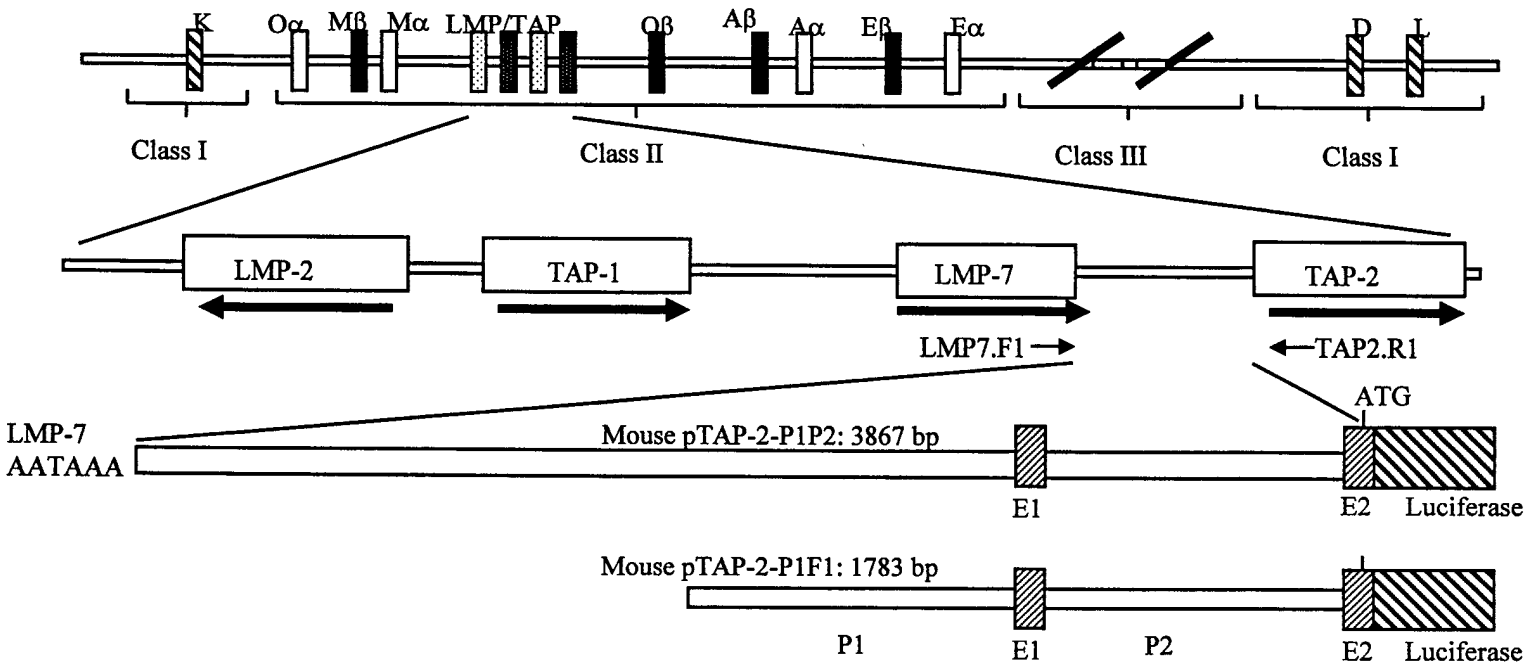
```
            >Exon 1 (TSS):
Genomic DNA: CGCGGGACCC AGGCGCGCTC CCCGCGAGGG CGTCGCTGCG CACCCAGGAG ATCCAGTTTG AGAAGAAGCA GATTCCAGAA
TAP2-1:      CGCGGGACCC AGGCGCGCTC CCCGCGAGGG CGTCGCTGCG CACCCAGGAG ATCCAGTTTG AGAAGAAGCA GATTCCAGAA

                                          >Intron 1
Genomic DNA: GCTCTCCTGA GCTGCCGCTC CGCAGCCGCA Ggtgagttct//.....//cacagacctc atttctccct ttctttccgc
TAP2-1:      GCTCTCCTGA GCTGCCGCTC CGCAGCCGCA G--------- ---------- ---------- ---------- ----------

              >Exon 2  +1
Genomic DNA: agAACCCACC ATGGCGCTGT CCTACCTGAG GCCCTGGGTC TCTCTGCTGC TGGCGGACAT GGCTTTACTT GGGTTGCTAC
TAP2-1:      --AACCCACC ATGGCGCTGT CCTACCTGAG GCCCTGGGTC TCTCTGCTGC TGGCGGACAT GGCTTTACTT GGGTTGCTAC
                                                                  <---------
                                                                  TAP2 Rev. Primer
```

Fig. 1

a. Mouse Chromosome 17 MHC Region



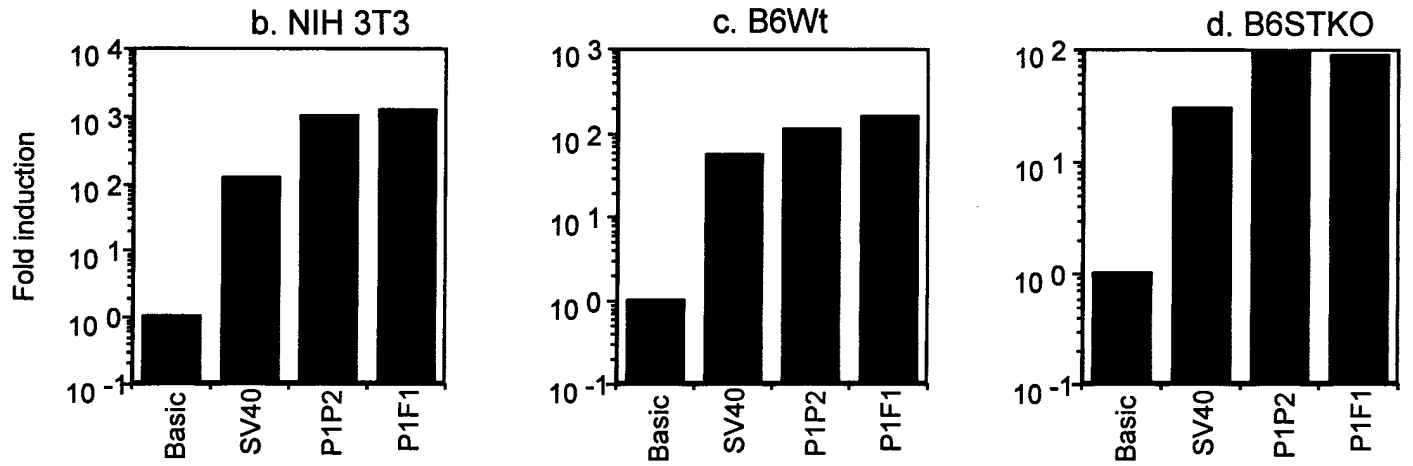
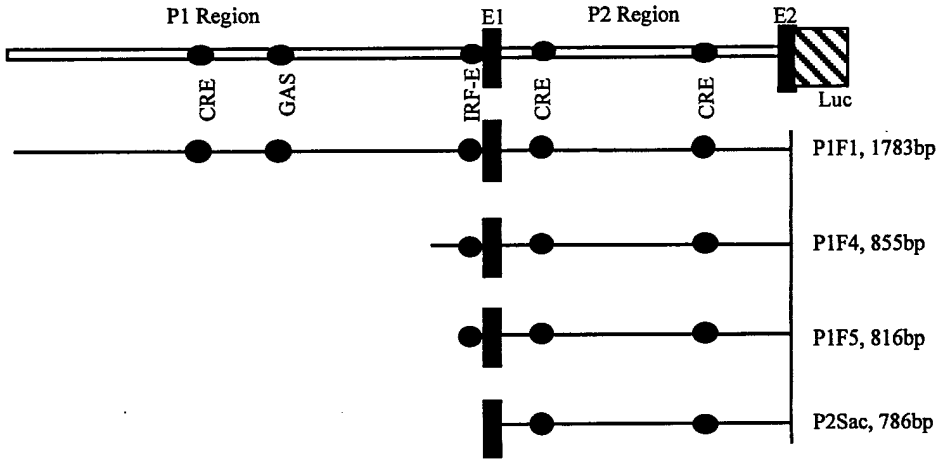


Fig. 2

a. DNA sequence of pTAP-2-P1F1:

```
          GAGT CATTTCTCCT TGTAGTGCCC TGGTGGTGGG GTGTGGTGAT GAGCAGCATG GTTGATTTTC ATGTCTGAGA ATGGAACCTG GTGCCTCACC ATGCTCTGTG AGGGCTCTTC
-1660 CCTGGAGCCC AGAAGGGTGA TTTCCGCAAT GAACTGGGCT CTGGGGAGGA GGCCGGGTTC CCTCTTTGAT GGTGTTAGCC TACAGCCTAC TCTGACTACC TCACAAGCAG CAGGCCCTGG
-1540 CACTGACCCA GGGCCCTCAC TGCAAGTGCG GTGTGCTGCG AAGAAAGTCT GAGGACAAAG GGCCAGAGCC TCTGGGTTCA AAATCTGTGA AAGGGAACCT TTGGCCTGCT GATAGGAGCT
-1420 GAGGCCTTCT TGTAAGCCCT AGTTTTACCC CAGACCCCAA CTCTTGAGCT TACCTGGAAA AACAAACAAG CCCTCAAGCC TCTCTTCTCT TCCACCCTCC GTCCTTCTCT CTCCTCTCTT
                                                          CRE
-1300 CCTTTTTCCA AAGGTGGGAC ACCTGTGTGG TCTGCTGACT GGGAGGGACC TGAAGTCACT CCCTCCTCCA TTTTCCTCCC TCCCCTTCCT TCCGTCCACG ACCATTCTCT TCCCTCGCCT
                                                          GAS
-1180 TTCTCCTTCT CCTATGCTCT TTTCTGCCCT TTACTCCTCC TCCTCTTTCC CCTTCCTCAA ACCTACTGGC AGCGAACCCT GGGCTCAGGC AAGTTTTCTC AACATCTGAA CTATAGTCCC
-1060 AGTTTAACCG GCGCTTCTTA AAACATGCTC GGAGGTCGGA GAAATGCAGG GTTTTTTCGT TTTTTTTTTT TGTTTGTTTT GTTTTTTTTT GTTTTTGTTT TTGTTTTTTT TTCCTTTTCA
-940  AAGCATCTCA GACCCTCAAG AGTTTTGCTA GTAGCGGCCT TGGAGGGCGA GCCTTTTTGG GAACCTGGGG CGCTCAACCC ACCCGCAAAT TGACAGGCGC CATCTGCTGG CGGCTGTGCG
                                  IRFE                    Exon 1
-820  CAGCACCGCG ACCACGGCAG TGAAGTGAAA GCGAAAGCCG CGGGACCCAG GCGCGCTCCC CGCGAGGGCG TCGCTGCGCA CCCAGGAGAT CCAGTTTGAG AAGAAGCAGA TTCCAGAAGC
                                                                                                                  CRE
-700 TCTCCTGAGC TGCCGCTCCG CAGCCGCAGG TGAGTTCTTT TGGATAGAAC CCGAGAGTTA GCAGGAGTGC GGCCCATTCC CAAACCCTTG GCGTCATCAT CCGGTTCCCC ACTCCTCGTC
-580  CCCTGTCTTG GGTCTTCTGC CGCTTACTCC CTGCCCCGTG TCCTCTGCTC CGTGCCCCTG CCCCACCCAG TGTCCCAGGT CCCCTGTCCC TCACCCCTTG CCCAAGGTCC TCTGCCCCAC
                                                                                                                  SP1
-460  CCCCTTGCCC TGCGTCCCCG GTCTTCCGTC GGGCGCCGCC GGCCCAGCGC CCCTACTCAG AACCTTCTGT CCCAAGCCAT CTAAGGCTAC CGAACCCACC CGCATCCCGC CCCTGCACCG
-340  GGTCTCCTGG CTCTTAAAGA CCCGAGGAAT GGGGGCAGTG TGTCTTCTTG GGCTAACACC AACAAACTTC AGGGAGATGG ATGTGGTGGC AGCAGACCGC CCCCTCCCCC ACCCCCACCC
                                                                    CRE
-220  CCGTGCTGTG TGTCTGAGTG TCTGTGTGTC TGTGTGACTG TGTGTGAGAA TAATGCAGCC TTCCCAGCTC CGTCAGACAG TCAGTCCTAG GAGAGACTTA TCTAGGAGGG GGTCTTTCCG
                                                                    Inr                Exon 2          TAP-2
-100  GTGAACAAGA AGCCTCAGGG TGGATTTGGG GCAGGAGGCT CGTAACCAGG GTTGGAACTC CACAGACCTC ATTTCTCCCT TTCTTTCCGC AGAACCCACC ATG
                                                                                                                  +1
```

b. pTAP-2 Promoter: P1 and P2 regions



c. Promoter activity

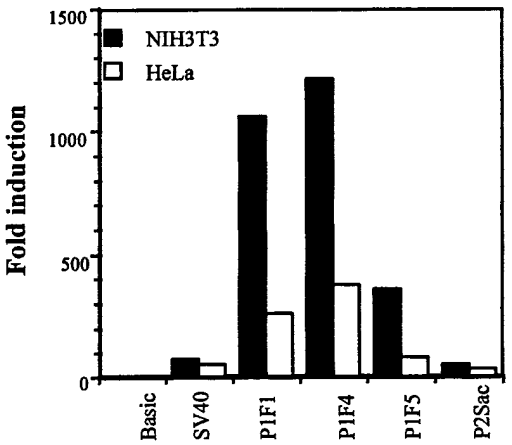
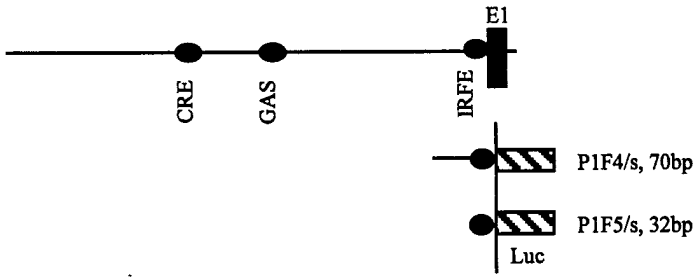


Fig. 3

a. pTAP-2 Promoter: P1 region



b.

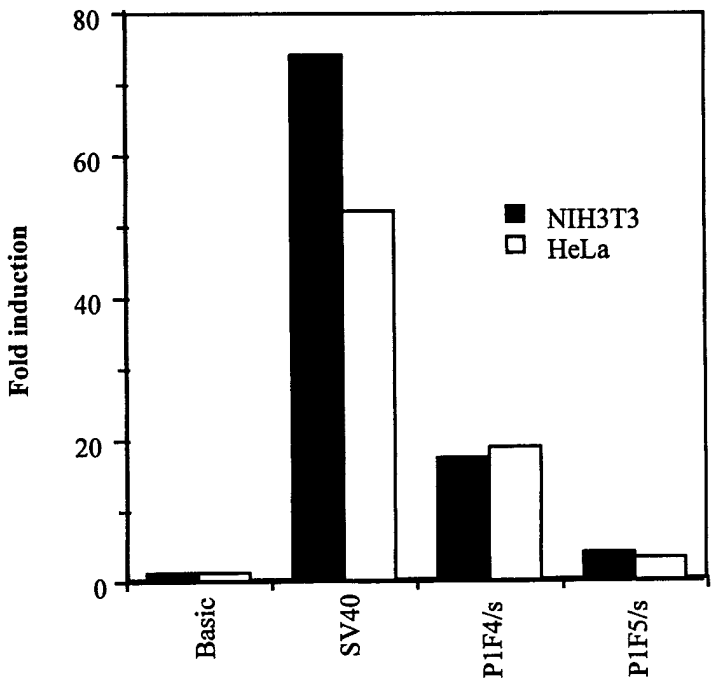
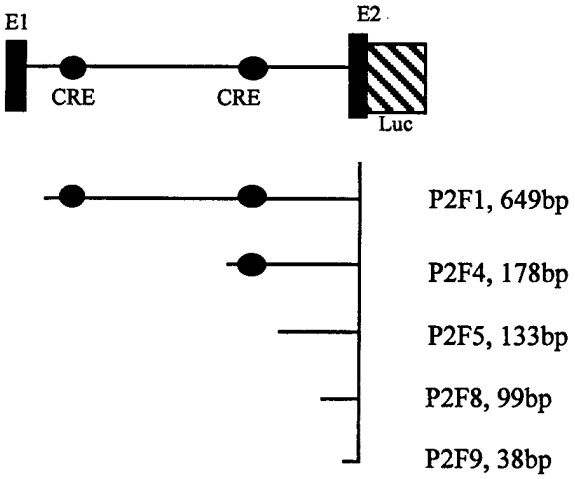
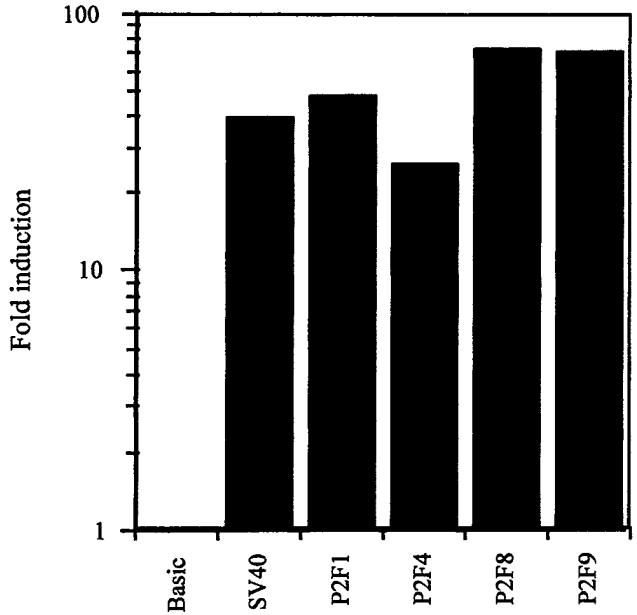


Fig 4

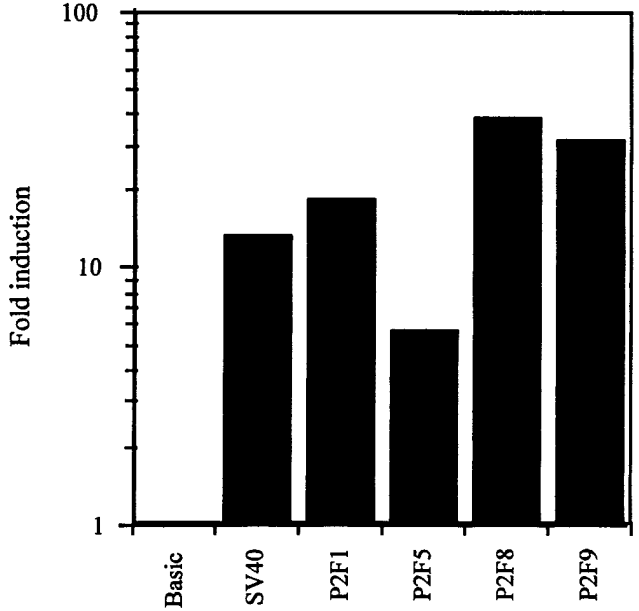
a. pTAP-2 Promoter: P2 regions



b. HeLa cells



c. NIH3T3



d. COS

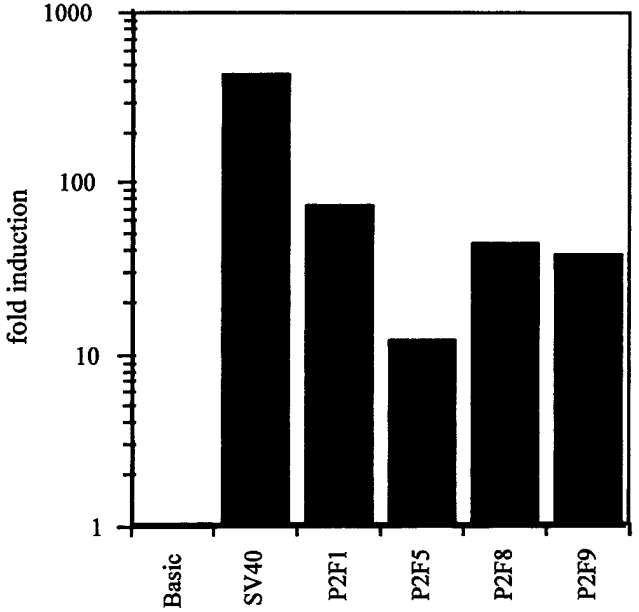


Fig. 5

a. Different TAP-2 transcription starting sites (TSS)

```
                >Exon 1 (TSS):
Genomic DNA:    CGCGGGACCC AGGCGCGCTC CCCGCGAGGG CGTCGCTGCG CACCCAGGAG ATCCAGTTTG AGAAGAAGCA GATTCCAGAA
TAP-2-1:        CGCGGGACCC AGGCGCGCTC CCCGCGAGGG CGTCGCTGCG CACCCAGGAG ATCCAGTTTG AGAAGAAGCA GATTCCAGAA
TAP-2-2:        ---------- ---------- ---------- --------CG CACCCAGGAG ATCCAGTTTG AGAAGAAGCA GATTCCAGAA
TAP-2-3:        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
TAP-2-4:        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                                                  >Intron 1                          Inr
Genomic DNA:    GCTCTCCTGA GCTGCCGCTC CGCAGCCGCA Ggtgagttct//.....//cacagacctc atttctccct ttctttccgc
TAP-2-1:        GCTCTCCTGA GCTGCCGCTC CGCAGCCGCA G--------- ---------- ---------- ---------- ----------
TAP-2-2:        GCTCTCCTGA GCTGCCGCTC CGCAGCCGCA G--------- ---------- ---------- ---------- ----------
TAP-2-3:        ---------- ---------- ---------- ---------- ---------- ---------- ATTTCTCCCT TTCTTTCCGC
TAP-2-4:        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                  >Exon 2  +1
Genomic DNA:    agAACCCACC ATGGCGCTGT CCTACCTGAG GCCCTGGGTC TCTCTGCTGC TGGCGGACAT GGCTTTACTT GGGTTGCTAC
TAP-2-1:        --AACCCACC ATGGCGCTGT CCTACCTGAG GCCCTGGGTC TCTCTGCTGC TGGCGGACAT GGCTTTACTT GGGTTGCTAC
TAP-2-2:        --AACCCACC ATGGCGCTGT CCTACCTGAG GCCCTGGGTC TCTCTGCTGC TGGCGGACAT GGCTTTACTT GGGTTGCTAC
TAP-2-3:        AGAACCCACC ATGGCGCTGT CCTACCTGAG GCCCTGGGTC TCTCTGCTGC TGGCGGACAT GGCTTTACTT GGGTTGCTAC
TAP-2-4:        ---------- ---------T CCTACCTGAG GCCCTGGGTC TCTCTGCTGC TGGCGGACAT GGCTTTACTT GGGTTGCTAC
```

b. Identification of TSS

No. of independent clones	PCR from cDNA Library	5'RACE From B6 Spleen	5'RACE From B6STKO spleen
TAP-2-1	3		
TAP-2-2		0	1
TAP-2-3		3	2
TAP-2-4		1	2

c. Identification of TSS by 5'RACE, followed by Southern Blotting.

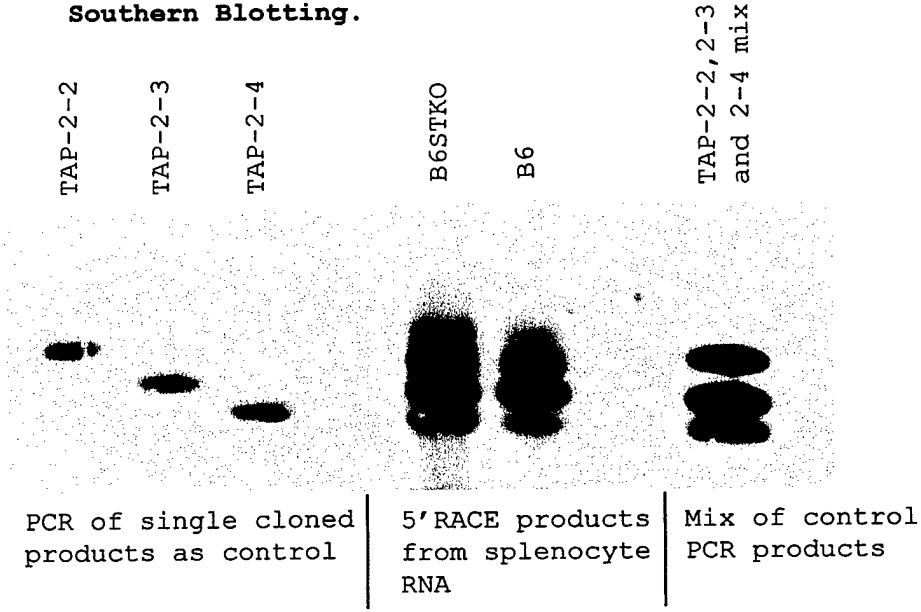
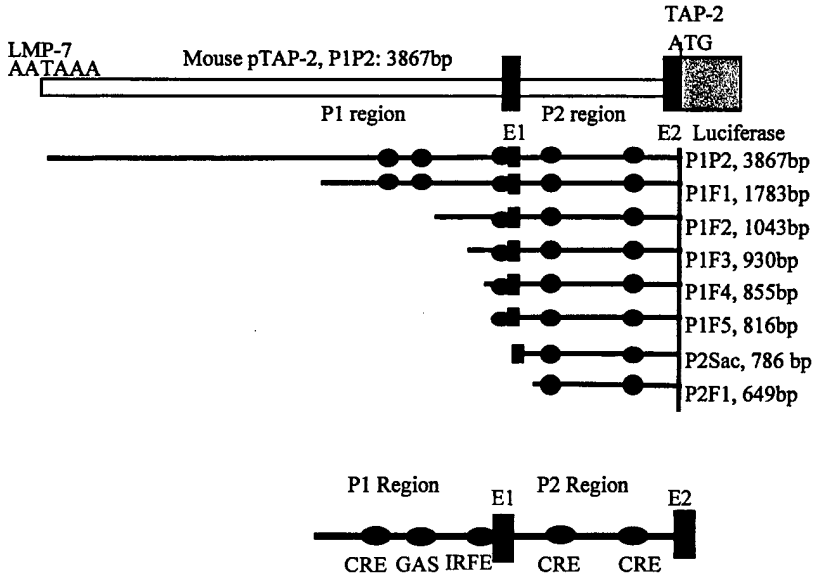


Fig. 6

a. Diagram of constructs



b. IFNγ induction

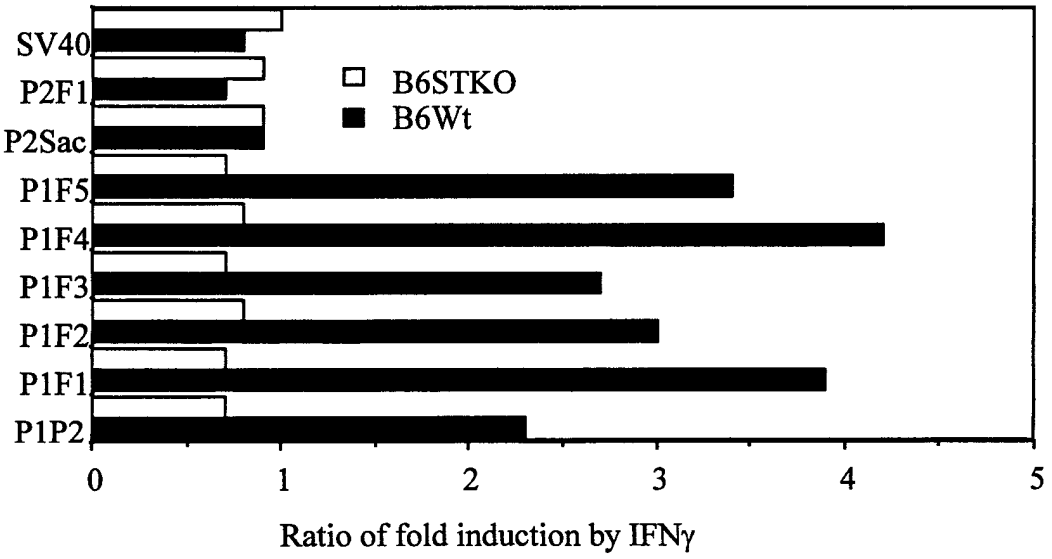


Fig. 7

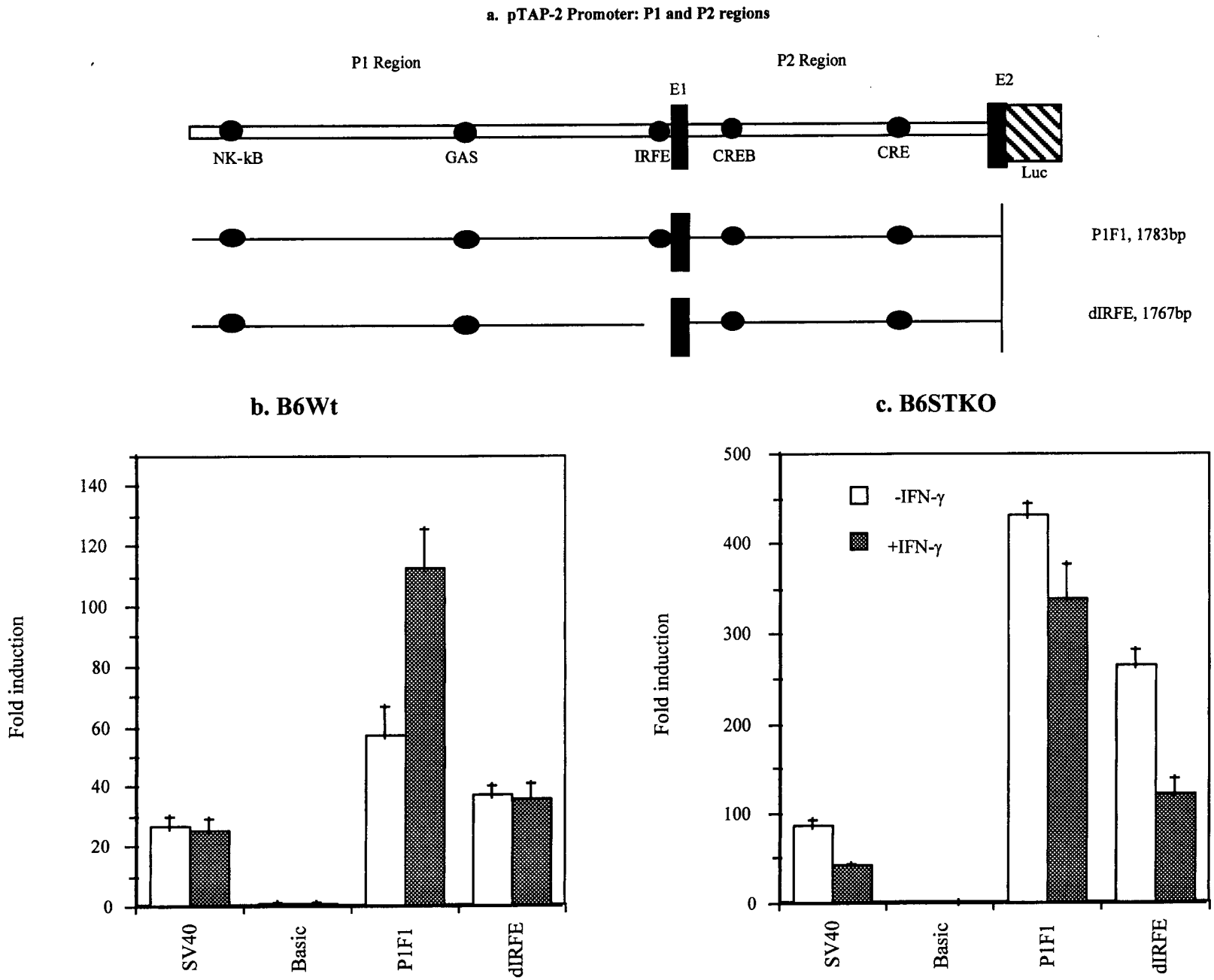
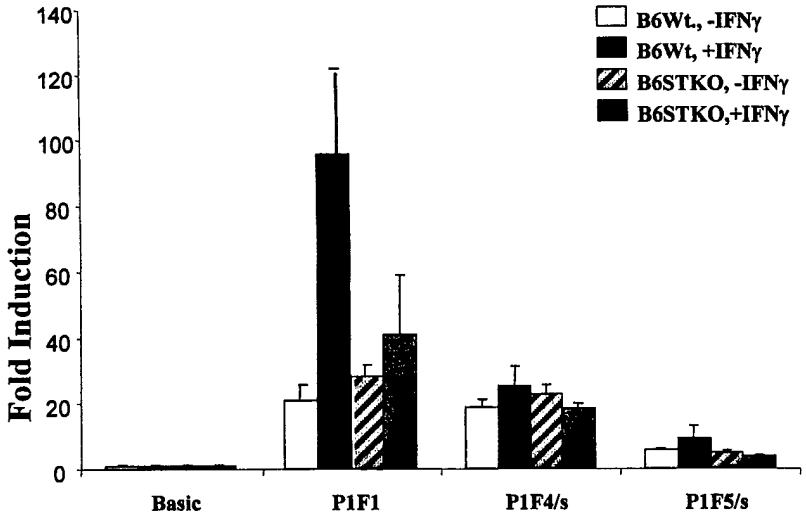

a. pTAP-2 Promoter: P1 and P2 regions
P1 Region
P2 Region
E1
E2
NK-kB
GAS
IRFE
CREB
CRE
Luc
P1F1, 1783bp
dIRFE, 1767bp
b. B6Wt
c. B6STKO
Fold induction
-IFN-γ
+IFN-γ
SV40
Basic
P1F1
dIRFE

Fig. 8

a. IFN-γ activates P1P2 but not P1



b. IRFE as the P2 enhancer

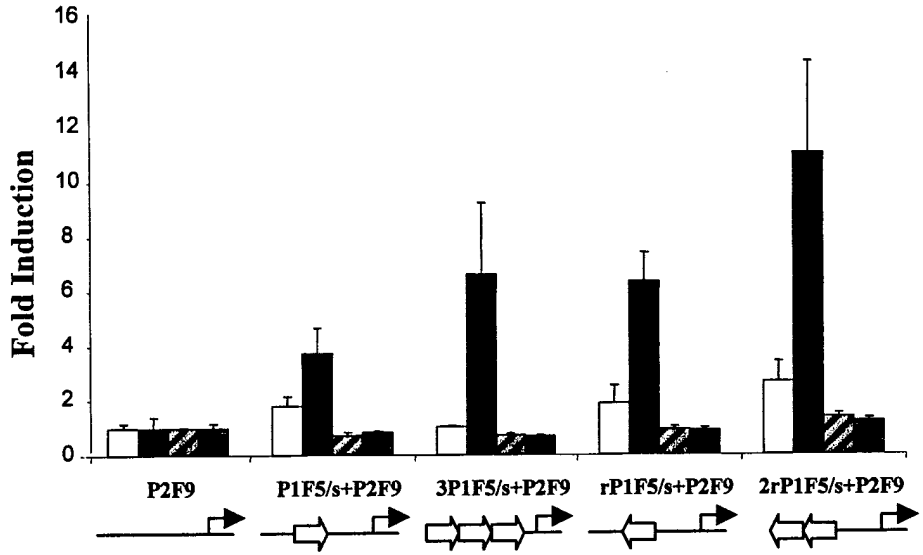


Fig. 9

Clonal deletion of SV40 large T antigen-specific T cells in the TRAMP mice: an important role for negative selection in shaping the repertoire of T cells specific for antigens over-expressed in solid tumors

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Running Title: Thymic clonal deletion in TRAMP mice

Keywords: immune tolerance, T cell development, tumor associated antigens, clonal deletion, prostate cancer model.

Total word count: 7675 words.

Summary

In addition to their over-expression in cancer cells, most of the tumor-associated antigens are expressed at low but detectable levels in normal tissues. It is not clear whether the repertoire of T cells specific for unmutated tumor antigens has been shaped by negative selection during T cell development. The transgenic adenocarcinoma of mouse prostate (TRAMP) model is transgenic for the SV40 large T antigen (Tag) under the control of the rat probasin regulatory elements. While it has been established that T lymphocytes from TRAMP mice are tolerant to SV40 Tag, the mechanism of the tolerance is largely unknown. To examine whether the T cell clonal deletion is responsible for the tolerance, we crossed the TRAMP mice with mice transgenic for a rearranged T-cell receptor specific for SV40 Tag presented by the H-2K^k. Double transgenic TRAMP/TCR mice show profound thymic deletion of SV40 Tag reactive T cells, including a 6-10 fold reduction in the total thymocyte numbers and a greater than 50-fold reduction in phenotypically mature T cells. Consistent with this finding, we observe that the SV40 Tag is expressed in a small population of thymic dendritic cells. These results demonstrate that clonal deletion is a major mechanism for tolerance to antigens previously regarded as prostate-specific, and thus provide the direct evidence that the T cell repertoire specific for an unmutated tumor antigen can be shaped by negative selection in the thymus.

Introduction

The majority of the tumor antigens identified so far have the same sequences as the endogenous genes (1-7). These unmutated tumor antigens are often recognized by T cells from cancer patients (8-13). With the notable exception of the PR-1 antigen in chronic myeloid leukemia (10), however, cancers appear to progress in spite of significantly expanded CD8 T cells specific for the tumor antigens. It has been suggested that these T cells are either anergic as a result of peripheral tolerance (9) or have low avidity for the cancer antigens (14). In the model of experimental autoimmune encephalomyelitis, it has been documented that T cells in mice lacking the auto-antigen, myelin basic protein, have an increased avidity for this antigen (15). Similarly, mice with a targeted mutation of tumor suppressor gene *p53* (16) or spontaneous mutation to tyrosinase (17) have T cells with intrinsically higher affinities than T cells from the wild-type mice. Therefore it is likely that tolerance to self-antigens may have removed T cells of high avidity for the unmutated tumor antigens. However, it is unclear whether high avidity T cells are removed by thymic clonal deletion or by mechanisms of peripheral tolerance.

Sarma et al examined the negative selection of T cells specific for unmutated tumor antigens using mice transgenic for antigen-specific TCR (18). The results indicated that transgenic T cells specific for the unmutated tumor antigen P1A can develop normally unless the tumor antigen is transgenically over-expressed in the thymus. However, since the transgenic TCR was isolated from a CTL clone that had gone through negative selection in mice that expressed the tumor antigen at low levels,

it is not surprising that transgenic T cells escaped clonal deletion. Thus, this work did not address whether the repertoire of T cells specific for unmutated tumor antigen is subjective to negative selection in the thymus. For this purpose one must start with a TCR isolated from mice lacking its specific antigen, and investigate the fate of T cells in mice that express this antigen.

SV40 large T antigen (Tag) is a potent oncogene. Tissue-specific expression of SV40 Tag leads to development of tissue-specific cancers, including cancers in liver (19), brain (20, 21), bone (22), and pancreas (23). Recently, Greenberg and collaborators have described a transgenic mouse model for prostate cancer: TRAMP (transgenic adenocarcinoma mouse prostate) mouse (24). In this model, a minimal rat probasin promoter regulatory element sequence was used to target expression of SV40 Tag to the epithelium of the mouse prostate.

Since the CTL epitopes for the large T antigens are among the most extensively characterized (25-28), many groups have used SV40 large T antigen transgenic mice directed by various promoters as models to study T cell tolerance to tumor-associated antigen (29-31). Functional analysis of different lines revealed that transgenic mice expressing SV40 Tag in several tissues including prostate are tolerant of the antigen (29-32). The tolerance is characterized by significantly reduced numbers and avidity of T cells upon immunization with either intact Tag or minigenes bearing the SV40 epitopes. In one model of osteosarcoma, tolerance developed over time with distinct kinetics for different epitopes (31). In none of these tumor models, however, has the mechanism for immune tolerance been clearly elucidated.

Self-antigen can induce tolerance by either central or peripheral tolerance. Central tolerance is characterized by clonal deletion (33, 34) or clonal anergy (35) of immature T cells in the thymus, while peripheral tolerance can be mediated by a number of mechanisms including clonal anergy (36), activation-induced cell death (37, 38), and regulatory cells (39). The mechanism of T cell tolerance to Tag in the TRAMP model is not clear, although it has been speculated that peripheral tolerance may be responsible (32) because previous studies have failed to detect Tag expression in non-prostate tissues including the thymus (32, 40). In this study, we crossed the TRAMP mice with TG-B mice (41), which are transgenic for a rearranged T-cell receptor that recognizes Tag presented by H-2K^k. We found that double transgenic TRAMP/TCR mice had strong thymic clonal deletion of SV40 Tag reactive T cells. These results provide the first direct evidence that tumor antigens perceived to be expressed exclusively in cancerous tissue can induce deletion of antigen specific T cells in the thymus.

Materials and methods

Experimental animals: C57BL/6 mice and TRAMP mice expressing the SV40 Tag controlled by rat probasin regulatory elements in C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred at the animal facilities of the Ohio State University (Columbus, OH). The production and characterization of transgenic mice (TG-B, B10.BR) expressing a rearranged T cell receptor of a $CD8^+$ cytotoxic T cell clone that recognizes SV40 Tag peptide 559-576 presented by the MHC class I molecule $H\text{-}2K^k$ were described previously (41, 42). The TG-B mice were bred to B10.BR mice at the animal facilities of the St. Jude's Children's Hospital and the Ohio State University.

Mouse genotyping: Mice were typed for SV40 Tag or TCR expression by isolation of mouse tail genomic DNA. The PCR-based screening assay were described previously (40, 43). TRAMP mice typing forward primer rPB.A: 5'-CCGGTCGACCGGAAGCTTCCACAAGTGAATTTA-3'; reverse primer rPB.B: 5'-AGGCATTCCACCACTGCTCCCATTCATC-3' (40); TG-B mice typing forward primer TG-B.aF: 5'-CCCTCATTGTCCCAGAGGGAGCCATGAC-3'; reverse primer TG-B.aR: 5'-CCCCCTCCGAATGTGAGCTTGGCACCTGC-3' (43). TG-B mice were also identified by tail bleeding and FACS analysis, by staining with FITC conjugated anti-Vb8.1+8.2 specific mAB (MR5-2) and PE conjugated anti-CD8 mAB (53-6.7).

Antibodies and cell lines:

The antibodies include anti-CD4 (RM4.5), anti-CD8 (53-6.7), anti-Vβ8.1+8.2 (MR5-2), anti- SV40 Tag (Pab 101) were purchased from BD Pharmingen (San Diego, CA). The

anti-CD11c supernatant was from the culture of hybridoma cell line HB224 purchased from ATCC (Manassas, VA).

The L929 cell line (H-2^k) was grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 4 mM L-glutamine (Gibco-BRL, Grand Island, NJ),

Synthetic peptides:

All peptides used were synthesized by Research Genetics, Inc. (Huntsville, AL). The peptides were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml and diluted in PBS or culture medium before use. Peptides used in these experiments were corresponded to SV40 Tag epitope IV 405-411(H-2 K^b restricted): VVYDFLKC (28); SV40 Tag 559-576 (H-2 K^k restricted): RSEFLLEKRIIQSGIALL (42); SV40 Tag 560-568 (H-2 K^k restricted): SEFLLEKRI (this report); and HSV gB peptide (H-2 K^b restricted) gB498-505: SSIEFARL (44).

Analysis by reverse transcriptase (RT)-PCR of total RNA extracted from tissue:

The thymus and spleen tissues, male urogenital organ complex including prostate tissue were harvested from mice, ages ranging from 2 days to 8 weeks old as indicated in text. Total RNA was extracted by TRIzol reagent according to the manufacturer's instructions (Gibco BRL). RNA concentration was determined and 1 μg of RNA was used to synthesize the first strand DNA by reverse transcriptase with the Superscript II Kit (Gibco BRL). The PCR reactions were carried out as 94°C 2 min, followed by 92°C 1 min, 55°C 1 min, 72°C 1 min for 35 cycles and extension of 72°C for 20 min. The SV40 Tag forward primer: SV40.F: 5'-TGGACCTTCTAGGTCTTGAAAGGAG-3'; reverse primer: SV40.R: 5'-AGGACTTCCACCACTGCTCCCATTCATC-3' (40); the ribosome L-

19 forward primer: L-19.F: 5'-CTGAAGGTCAAAGGGAATGTG-3'; reverse primer: L-19.r: 5'-GGACAGAGTCTTGTGATCTC-3' (40), the murine probasin forward primer: mPB.F: 5'-ATCATCCTTCTGCTCACACTGCAT-3', reverse primer: mPB.R: 5'-ACAGTTGTCCGTGTCCATGATACGC-3' (45). PCR products were separated by 1.5% agarose gel electrophoresis, transferred to a Hybond N+ membrane (Amersham Pharmacia Biotech, Inc. Piscataway, NJ). The membrane was hybridized with DNA probes and signals were detected by ECL direct nucleic acid labeling and detection system according to the manufacturer's instructions (Amersham Pharmacia Biotech Inc.).

Peptide immunization and ELISpot assay:

C57BL/6 or TRAMP mice of 8-12 wks of age were immunized subcutaneously with 100 μg SV40 Tag epitope IV peptide in complete Freund's adjuvant (400 μg of mycobacterium tuberculosis per ml) in a total volume of 100 μl PBS. Draining lymph nodes and spleens were isolated at 9 days after immunization. ELISpot assays were performed to evaluate frequencies of T cells that produce IFN-γ upon restimulation with SV40 Tag epitope IV in vitro according to the manufacturer's instructions (BD Pharmingen). An unrelated antigen, HSV gB peptide, was used as non-specific peptide control. A six-point titration of cells was applied to the culture containing either 1 μg/ml antigen specific peptide SV40 Tag epitope IV or 1 μg/ml control peptide HSV gB epitope. The spots were counted under a contrast microscope. After subtracting the non-specific IFN-γ spots produced by HSV gB stimulation from the SV40 Tag epitope IV IFN-γ spots, the linear range regression analysis was used to obtain the antigen specific spots per $1x10^6$ splenocytes or lymph node cells.

Proliferation of T cells to antigenic peptides and CTL assays.

Total spleen cells ($3x10^5$/well) from TRAMP x TG-B ($H\text{-}2^{bxk}$) F1 mice were cultured with the given concentrations of SV40 Tag K560-568 peptide or control HSV gB peptide in Click's EHAA medium for 72 hours. The proliferation of T cells was determined by incorporation of ^{3}H-Thymidine (TdR) pulsed (1 μCi/well) during the last 6 h of culture. The data presented are means of duplicates with variation from the means < 15%.

In CTL assay, the total spleen cells from TRAMP x TG-B ($H\text{-}2^{bxk}$) F1 mice were cultured in 0.1 μg/ml SV40 Tag 560-568 peptide in Click's EHAA medium for 5 days and used as effector cells. As target cells, we used L929 ($H\text{-}2^K$) pulsed with either SV40 Tag 560-568 peptide or control HSV gB peptide. These targets were labeled with ^{51}Cr for 1 h at 37°C. The effector cells and target cells were added to the 96-well plate at different E/T ratios. After 6 h, the released ^{51}Cr in the supernatants was harvested and determined by a Packard TopCount NXT Microplate Scintillation and Luminescence Counter. The specific percentage of lysis was calculated as: Specific % lysis = $[(cpm_{samples} - cpm_{medium})/(cpm_{maxim} - cpm_{medium})] \times 100$. The data presented are means of duplicates.

Histology and Immunohistochemistry study:

Freshly harvested mouse thymus, spleen and prostate tissues were snap frozen in isopentane and stored at –70°C. 5-μm thick sections were cut with a cryostate (Microm HM 505E, Fisher Scientific, Fairlawn, NJ) at –20°C, and frozen sections were fixed with acetone prior to staining with hematoxylin and eosin (H&E). For double label immunostaining, we used VECTASTAIN Elite ABC kits following the protocol provided by manufacturer (Vector Laboratories, Burlingame, CA). Briefly, the frozen sections were fixed in cold acetone and blocked with 1% H_2O_2, avidin blocking solution, and

biotin blocking solution (Vector Lab.), respectively. This was followed by preincubation with 5% normal horse serum in PBS for 30 min. The sections were then incubated with mAb to SV40 Tag (Pab 101, BD Pharmingen) for 1 h. The biotinylated horse anti-mouse IgG was applied to the slides as a secondary antibody, followed by application of avidin-biotin-peroxidase-antiperosidase complex (ABC, Vector Laboratories). The Vector VIP (purple) (Vector Lab.) was used as first step enzyme substrate. The sections were treated with 1N HCl for 10 min and preincubated with 10% goat normal serum for 10 min. The sections were then incubated with hamster anti-mouse CD11c (HB224) supernatant for 1 h. The biotinylated goat anti-hamster IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was applied to the slides as a secondary antibody, followed by application of ABC reagents. Diaminobenzidine (DAB, Vector Lab.) was used as a second step enzyme substrate. All sections were counterstained in Vector's Methyl Green Counterstain (Vector Lab.) and then mounted with Permount histologic mounting medium (Fisher Scientific). Histological and immunostained slides were studied and photographed using an Olympus BX-40 microscope.

Results

Both male and female TRAMP mice are tolerant to an immunodominant CTL epitope in SV40 Tag.

Male TRAMP mice developed immune tolerance against the SV40 Tag as they fail to respond when immunized with SV40 Tag transfected fibroblast B6-3T3 cells (40). As the first step to determine whether prostate expression of Tag is responsible for the tolerance, 8 to 12 week old male or female C57BL/6 background TRAMP mice and their Tag-negative littermates were immunized subcutaneously with 100 μg of MHC H-2K^b restricted immunodominant Tag epitope IV peptide (residue 404-411) (46) in complete Freund's adjuvant between 8 to 12 weeks of age. After 9-10 days, the mice were sacrificed and draining lymph node cells and splenocytes were collected. The frequency of Tag epitope IV specific IFN-γ producing cells were determined by ELISpot assay. An unrelated H-2K^b peptide from Herpes Simplex virus (HSV-1) gB peptide was used as control (44). As shown in Fig 1, Tag-negative male mice (Tag- Imm) developed vigorous response to immunodominant Tag epitope IV, while the male TRAMP mice produced a barely detectable T cell response, consistent with prior studies that revealed immune tolerance to this epitope in male TRAMP mice (32). Surprisingly, the number of Tag-specific T cells was also low in female TRAMP mice in comparison to Tag-negative littermates. Thus, TRAMP female mice are also tolerant to this antigen. These results indicate that expression of Tag in the prostate is not required for induction of tolerance to Tag in TRAMP mice.

Thymic clonal deletion of transgenic T cells in TRAMP/TG-B double transgenic mice.

TG-B mice express a rearranged T cell receptor from a $CD8^+$ cytotoxic T cell clone that recognized SV40 Tag presented by MHC class I molecule $H\text{-}2K^k$ (10). The presence of transgenic T cells can be monitored by flow cytometry with anti-CD8 and anti-$V_\beta 8$ specific monoclonal antibodies. To test whether the tolerance was caused by thymic clonal deletion of SV40 Tag reactive T cells, we bred the TRAMP mice with TG-B mice to obtain TRAMP/TG-B double transgenic and TG-B single transgenic F1 ($H\text{-}2^{bxk}$) mice. The fates of SV40 Tag specific T cells in the central and peripheral lymphoid organs were examined in the F1 mice at 25 to 30 days after birth. Gross anatomic examination showed that the thymi of TRAMP/TG-B double transgenic mice were wrinkled and much smaller than those of non-transgenic or TG-B single transgenic mice. This was consistent with the number of viable cells recovered from the thymus (Fig. 2a). In male double transgenic mice, the total thymocytes were $1.1x10^7$ cells in average compared to $6.5x10^7$ cells in TG-B single transgenic F1 mice. Thymocytes were then stained with anti-CD4, anti-CD8 and anti-$V_\beta 8$ antibodies and examined by three-color flow cytometry. The upper panels in Fig. 2b depict the composition of the thymocytes of the male mice, while the lower panels show those of the female mice. The percentages of $CD4^+CD8^+$ cells and $CD8^+CD4^-$ cells were significantly decreased in TRAMP/TG-B double transgenic mice in comparison to those in TG-B single transgenic mice. The female double transgenic mice showed more profound decrease in these two populations than the male mice. Among the $V_\beta 8^{high}$ cells, $CD8^+CD4^-$ cells were reduced by greater than 50-fold in the thymi of TRAMP/TG-B double transgenic mice (Fig. 3a). The majority (74%) of $V_\beta 8$

positive cells were $CD4^-CD8^-$ (Fig.3b, upper right), which is consistent with clonal deletion occurring at the early $CD4^+CD8^+$ stage of developments as has been seen in other MHC class I restricted TCR-transgenic mice (33, 34). These data therefore demonstrate that the SV40 Tag-specific transgenic T cells are efficiently deleted in the TRAMP/TG-B double transgenic mice. Surprisingly, the clonal deletion appears more complete in female double transgenic mice than males, as suggested by more severe reductions in the proportion of $CD4^+CD8^+$ and $CD8^+CD4^-V\beta8^{high}$ T cells in thymus (Fig. 2 and Fig.3).

Corresponding to the clonal deletion in the thymus, we also found that the T cells reactive to SV40 Tag were selectively depleted in peripheral lymphoid organs. Among splenic T cells, the ratio of CD4/CD8 single positive cells was 2:1 in non-transgenic mice and 1:4 in TCR single transgenic mice (data not shown). As expected, the majority of $CD8^+$ T cells expressed $V_\beta8$ in the TCR single transgenic mice (Fig. 4a, left panels). However, in double transgenic mice, the absolute numbers of T cells were significantly decreased and the $CD8^+V\beta8^+$ cell population was absent (Fig.4a, right panel).

Previous mapping of the $H\text{-}2K^k$ restricted cytotoxic T lymphocyte epitope in SV40 Tag using in-frame deletion mutants mapped the epitope in the position of peptide 559-576 in large T antigen (21), which is significantly longer than the known size of peptides presented by MHC class I. We compared this 18-residue-peptide with consensus sequence of $H\text{-}2K^k$-binding peptide (47) and synthesized a 9-residue peptide (560-568) with a minimal motif: SEFLLEKRI. As shown in Fig. 4b, the peptide Tag 560-568 was capable of inducing transgenic T cell proliferation from TCR single transgenic F1 mice. After 5 days of in vitro stimulation with Tag peptide 560-568, the activated transgenic T

cells lysed L929 cells pulsed with peptide (Fig. 4c). In contrast, the splenocytes from the double transgenic mice did not proliferate when stimulated with SV40 Tag peptide 560-568 (Fig. 4b). In addition, after 5 days of in vitro stimulation, TRAMP/TG-B splenocytes failed to lyse Tag peptide-pulsed L929 cells (Fig. 4c). Taken together, both flow cytometry and functional assays demonstrated that clonal deletion of $CD8^+V\beta8^+$ cells is almost complete in both male and female double Tag^+/TCR^+ transgenic mice.

Thymic expression of SV40 Tag and murine probasin in TRAMP mice.

Previous studies generally support the notion that the SV40 Tag is expressed exclusively in the prostate in TRAMP mice, starting at 4 week after birth (40, 48, 49). However, the fact that strong clonal deletion was observed even in female mice cast doubt on the exclusivity of Tag expression in the prostate. Since we were also unable to reproducibly detect Tag expression in the thymus from the TRAMP mice by routine Northern blot or RT-PCR method, we increased the detection sensitivity by performing the RT-PCR followed by Southern Blotting. This allowed reproducible detection of Tag expression in the thymus. Contamination of DNA was excluded by two criteria: first, the size of products were as predicted after RNA splicing (260 bp). Second, no product of this size was detected unless the reverse transcriptase was used. As shown in Fig 5a, by using this detection method, we were able to reproducibly detect SV40 Tag expression in the thymus as early as 2 days after birth. Before puberty, we found that the SV40 Tag expression level was higher in thymus than that in prostate gland (Fig 5b). The SV40 Tag expression was greater than 1000-fold increased in prostate gland after puberty, which is consistent with the androgen-mediated activation of probasin promoter (50). Interestingly, the thymic expression of SV40 Tag was not augmented in

this process (Fig. 5c). Thus, thymic and prostatic Tag expressions were differentially regulated.

The low levels of Tag expression in the thymus can be due to either poor expression on a large number of cells, or high expression on a small number of specialized cells. To address this issue, frozen sections of TRAMP mouse thymus were double immunostained with anti-Tag antibody Pab101 that reacted with the Tag located on nucleus and anti-CD11c antibody that stained the cell membrane. As shown in Fig. 6, essentially all cells expressing SV40 Tag also expressed CD11c, a marker for dendritic cells. However, more than 95% of the DC did not express Tag. Moreover, while the majority of the double positively stained cells were located in the medulla (M), some were scattered in the cortical-medullar region (J), and rare double positive cells could be found in cortex (data not shown). The presence of the Tag^+ cells at multiple sites explained the strong clonal deletion of Tag-specific T cells starting at the $CD4^+CD8^+$ stage.

To rule out the possibility that expression of Tag is an artifact of the transgene, we also tested if the endogenous probasin is expressed in the thymus. Mouse probasin has been cloned and its expression has been shown to be prostate specific (45). Using the same RT-PCR plus southern blot method, we found that murine probasin mRNA was detectable in the thymus of TRAMP mouse before puberty (25 days after birth), although the mPB expression was much stronger in the prostate (Fig. 7). Thus, expression of Tag is not a transgenic artifact, and is therefore representative of prostate specific genes.

Discussion

It has been reported that in TRAMP mice, T cells specific for SV40 Tag are tolerized (32). However, the mechanisms for the tolerance were not elucidated. Here we used what is now a classical double-transgenic approach to demonstrate that the Tag-specific T cells are deleted in the thymus. Since the Tag is expressed in the thymus, the simplest interpretation is that Tag expressed in these cells is directly responsible for clonal deletion, although it is still possible that the cell mediating clonal deletion has acquired Tag expressed elsewhere via cross-presentation. Nevertheless, equally efficient clonal deletion in female mice excluded the possibility that the prostate is the donor of this antigen.

It is worth noting that several groups have failed to detect Tag expression by both Northern blot and RT-PCR (40, 48, 49). While we have had similar experiences with these methods, we were able to detect Tag after we used Southern blot to increase the sensitivity of detection. The requirement for an additional step to reveal the Tag product suggests that the Tag is expressed at low levels in the thymus. However, immunohistochemistry revealed that the overall low abundance of Tag mRNA in the thymus is likely due to a small number of Tag-expressing cells. The significant levels of Tag expression among the cells known to be involved in clonal deletion (DC in medulla and the medulla junction) support their role in deletion of the Tag-specific T cells.

Expression of Tag in a small subset of DC in the thymus is not surprising. Tag, under the control of at least two other tissue-specific promoters (insulin for β-cell expression and elastase I for pancreatic acinar cell expression) (51, 52) was also

reported to be expressed in the thymus. The expression of Tag was not a transgenic artifact as other pancreatic genes were also found in the thymus (52). Most strikingly, genetic variations of insulin expression in the thymus may account for differential susceptibility to type II diabetes in human (53, 54). These observations lead Hanahan to propose a specialized population(s) of cells in the thymus that express antigens previously regarded as tissue-specific, which were termed PAE (peripheral antigen-expressing) cells (55). However, while thymus transplantation studies implicate a role for PAE in inducing tolerance (51, 52), clonal deletion has not been clearly established in transgenic mice expressing both TCR and the antigen in PAE (51, 56). The failure to observe consistent tolerance was attributed to low number of PAE (estimated to be within 100-300/thymus or approximately one cell per thymic section). To our knowledge, our study is the first example of complete clonal deletion in the thymus that may be attributable to expression of antigen in PAE. This is probably due to the fact that the number of Tag^+ PAE is significantly higher than the numbers reported in the other models, as we observed 10-13 cells per thymic section.

In several Tag-induced spontaneous tumor models, including cancers developed in the liver (29), brain (30); bone (31), and prostate (32), it has been reported that active immunizations fail to induce protection. Correspondingly, T cell response to immunization by various forms of Tag is either absent or of extremely low avidity (29-32). Since transfer of either naive or activated T cells from Tag-negative mice can convey partial or full protection, T cells in these various Tag-transgenic mice must have been rendered tolerant to Tag. Our study suggests that thymic expression of genes so-

called tissue-specific genes caused clonal deletion of T cells specific for the tumor antigen.

Since unmutated tumor antigens are present in a high proportion of cancers of the same histological origin as well as those that are from different lineages (1-7), these antigens are the primary targets for immunotherapy. The nature of T cell repertoire to unmutated antigens is of both fundamental and practical significance. Using mice transgenic for a TCR specific for unmutated tumor antigen P1A, Sarma et al. reported that P1A-specific T cells are not deleted in wild-type mice with a low expression of P1A (18). At face value, this appears contradictory to the finding with Tag-specific T cells, as reported here. However, it is important to bear in mind that P1A-specific TCR was isolated in a CTL clone from mice that express the P1A gene (1, 18, 57). The very fact that these T cells can be produced indicates that this TCR is not deleted in wild-type mice. As such, the previous work did not address whether the repertoire of T cells specific for unmutated tumor antigens has been shaped by negative selection in the thymus. In contrast, the TCR for TG-B was isolated from mice that do not express Tag (42). Therefore, the transgenic T cells are representative of unpurged Tag-specific T cells and can be used to address whether Tag expressed under a prostate specific promoter can significantly alter the Tag-specific T cell repertoire. Our results revealed almost complete deletion of Tag-specific T cells in TRAMP mice. Obviously, in mice with a polyclonal TCR repertoire, some T cells may escape clonal deletion. However, since clonal deletion removes T cells with high-affinity TCR for the antigen in the thymus (58), it is likely that high affinity Tag-specific T cells are removed from the naive T cell pool in the TRAMP mice. Thus strategies for immunotherapy of prostate cancer and

other cancers targeting unmutated tumor antigens must take into consideration that the T cell repertoire will have been depleted of the T cells of the highest affinity. In this regard, recent studies have demonstrated that supra-agonist antigenic peptides may help to overcome this problem (14, 59). Alternatively, one may attempt to prevent the deletion of T cells with high avidity for tumor antigen. It was realized recently that *de novo* production of T cells lasts throughout much of an organism's life span (60, 61). This raises an interesting possibility that strategies aimed at preventing the deletion of tumor antigen specific T cells will have a positive impact in cancer immunotherapy.

Acknowledgement

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Figure legend

Fig. 1. Both male and female mice are tolerant to SV40 Tag. Male and female TRAMP mice and their non-transgenic littermates were immunized with the major Tag epitope. At 9 days after immunization, the spleens and lymph nodes from naive and immunized mice were harvested and Tag-specific IFN-γ producing T cells were measured by Elispot Assay. Six-point titrations of cells were applied to the culture in 96-well plates containing either 1 μg/ml antigen specific peptide SV40 Tag epitope IV or 1 μg/ml control peptide HSV gB epitope. The spots were counted under a contrast microscope. The SV40 Tag antigen specific spots were calculated by subtracting the means of correlated non-specific HSV gB peptide IFN-γ spots from the means of SV40 Tag epitope IV peptide IFN-γ spots. Linear range regression analysis was used to obtain the antigen specific spots per 10^6 splenocytes or lymph node cells. Data shown are representative of three independent experiments.

Fig. 2. Clonal deletion of Tag-specific T cells in the thymus. Thymus tissues from SV40 Tag^+/TCR^+ double transgenic F1 mice and SV40 Tag^-/TCR^+ single transgenic F1 mice were harvested at day 25 after birth. Thymocytes were stained with anti-CD4 and anti-CD8 antibodies. The total thymocytes numbers (a) and the subset distribution of thymocytes (b) were presented. The numbers in the quadrants were % of cells. Data shown were from one representative of at least 2-3 mice per group in a total of four independent experiments.

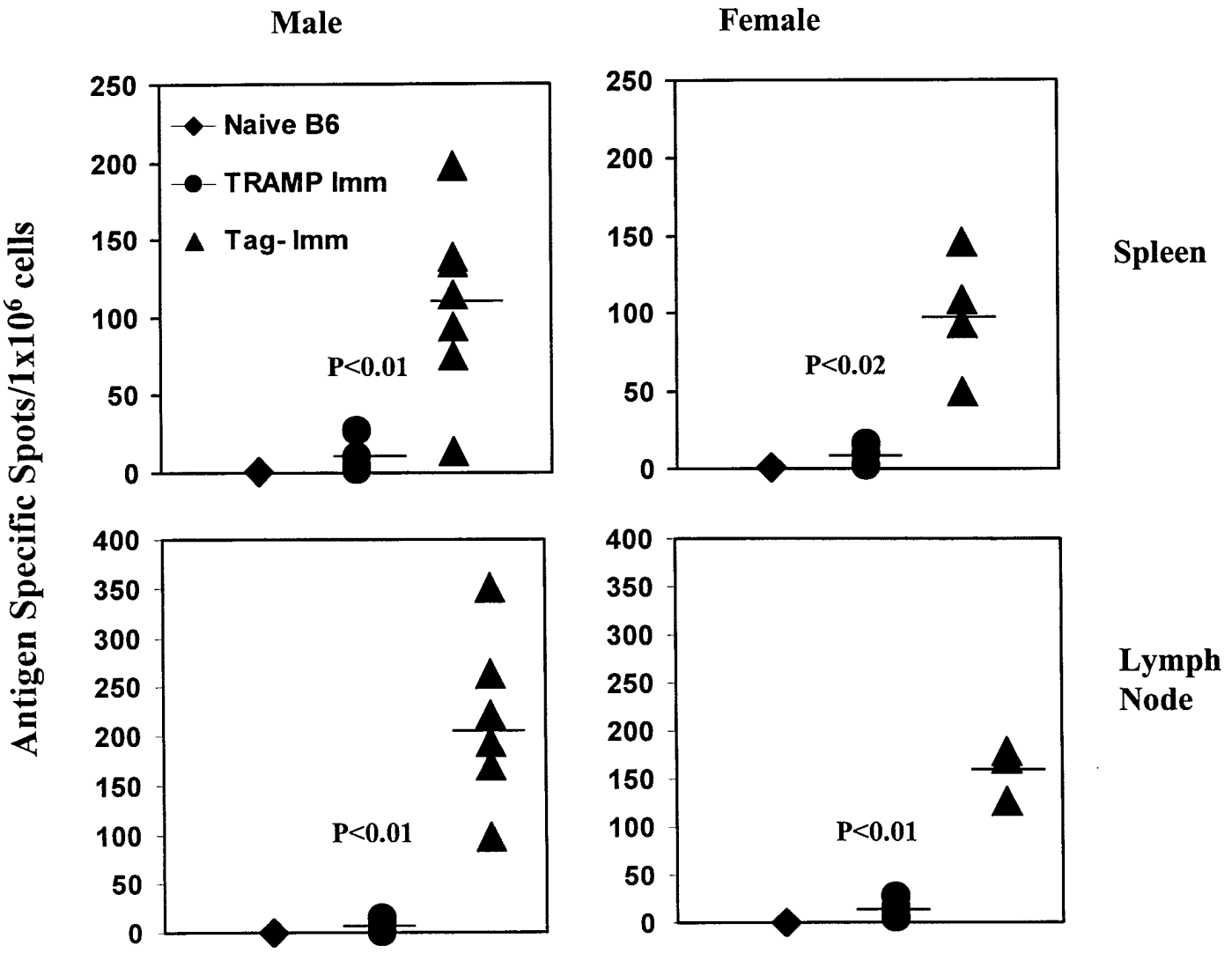
Fig. 3. Numbers and phenotypes of Tag-specific T cells. Thymus tissues from SV40 Tag^+/TCR^+ double transgenic F1 mice and SV40 Tag^-/TCR^+ single transgenic F1 mice were harvested at day 25 after birth. Thymocytes were stained with anti-CD4, anti-CD8 and anti-$V_\beta 8$ antibodies and examined by three-color flow cytometry. $V_\beta 8^{high}$ T cells were gated. (a). Numbers of $V_\beta 8^{high}CD8^+CD4^-$ cells in thymus. (b). The CD4/CD8 expression among the gated $V_\beta 8^{high}$ thymocytes. The numbers in the quadrants were % of cells. Data shown are from one representative of at least 2-3 mice per group in a total of four independent experiments.

Fig. 4. Numbers and functions of mature Tag-specific T cells in the spleen. Spleens from SV40 Tag^+/TCR^+ double transgenic F1 mice and SV40 Tag^-/TCR^+ single transgenic F1 mice were harvested at day 25 after birth. (a). Splenocytes were stained with anti-CD4, anti-CD8 and anti-$V_\beta 8$ antibodies and examined by three-color flow cytometry to detect the percentage of the $V_\beta 8^+CD8^+$ transgenic T cells. The numbers in the quadrants were % of cells. (b) Proliferation of Tag-specific T cells. Splenocytes were stimulated for 66 hours in the presence of Tag peptides 560-568 (solid lines) or control HSV gB peptides (broken lines) in different concentrations as indicated, and proliferation was detected by pulsing the culture with ^{3}H-TdR for 6 additional hours. (c). The cytotoxicity of Tag-specific T cells. Splenocytes were cultured with 0.1 μg/ml Tag peptide 560-568 for 5 days. Viable cells were purified by Ficoll reagent and used as effectors. Tag-peptide pulsed (solid lines) or HSV gB peptide (broken lines) pulsed ^{51}Cr labeled L929 cells were used as target cells. Data shown are from one representative of five independent experiments.

Fig. 5. SV40 Tag expression in the thymus tissues and in the prostates as detected by RT-PCR followed by Southern blot. (a). Expression of SV40 Tag mRNA in thymus from mice of various ages. The first strand DNA (RT+) and RNA (RT-) were used as templates and primers for either Tag (35 cycles) or control L-19 gene (25 cycles) were used for PCR reactions. RT-PCR results of L-19 amplification were shown as agarose gel image. The SV40 Tag PCR products were separated by agarose gel electrophoresis and transferred to the Hybond N+ membrane. The membrane was hybridized with HRP-labeled probe and signals were detected by use of an ECL direct nucleic acid labeling and detection system. (b). Comparative analysis of Tag mRNA in thymus and prostate of pre-puberty mice (25 days). (c). Comparative analysis of Tag mRNA in thymus and prostate of post-puberty mice (8 weeks). The first strand DNA was diluted as indicated and PCR reactions were carried out, the SV40 Tag PCR was 35 cycles, and the L-19 PCR was 15 cycles. Both PCR products were subjected to Southern Blotting by ECL method described above. The films were exposed for either 30 sec. or 3 min for SV40 Tag, and 10 sec. for L-19.

Fig. 6. Detection of Tag expression in dendritic cells in the thymic medulla (M) and the junction of medulla and cortex (J). The frozen sections of TRAMP mouse thymus tissue were double immunostained with anti-SV40 Tag (Vector VIP substrate, purple) and anti-CD11c (DAB, brown) (upper left panel) or immunostained with anti-CD11c alone (lower left panel) in consecutive sections. Three representative double positive Tag expressing dendritic cells are shown in the right panels.

Fig. 7. The thymic expression of murine probasin. RT-PCR followed by Southern blot was carried out in the RNA sample isolated from a 25 day old TRAMP mouse. The experiments were performed as described in Fig. 5.


Male
Female
Antigen Specific Spots/1x10^6 cells
Naive B6
TRAMP Imm
Tag- Imm
250
200
150
100
50
0
P<0.01
P<0.02
Spleen
400
350
300
250
200
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100
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0
P<0.01
P<0.01
Lymph Node

Fig.1

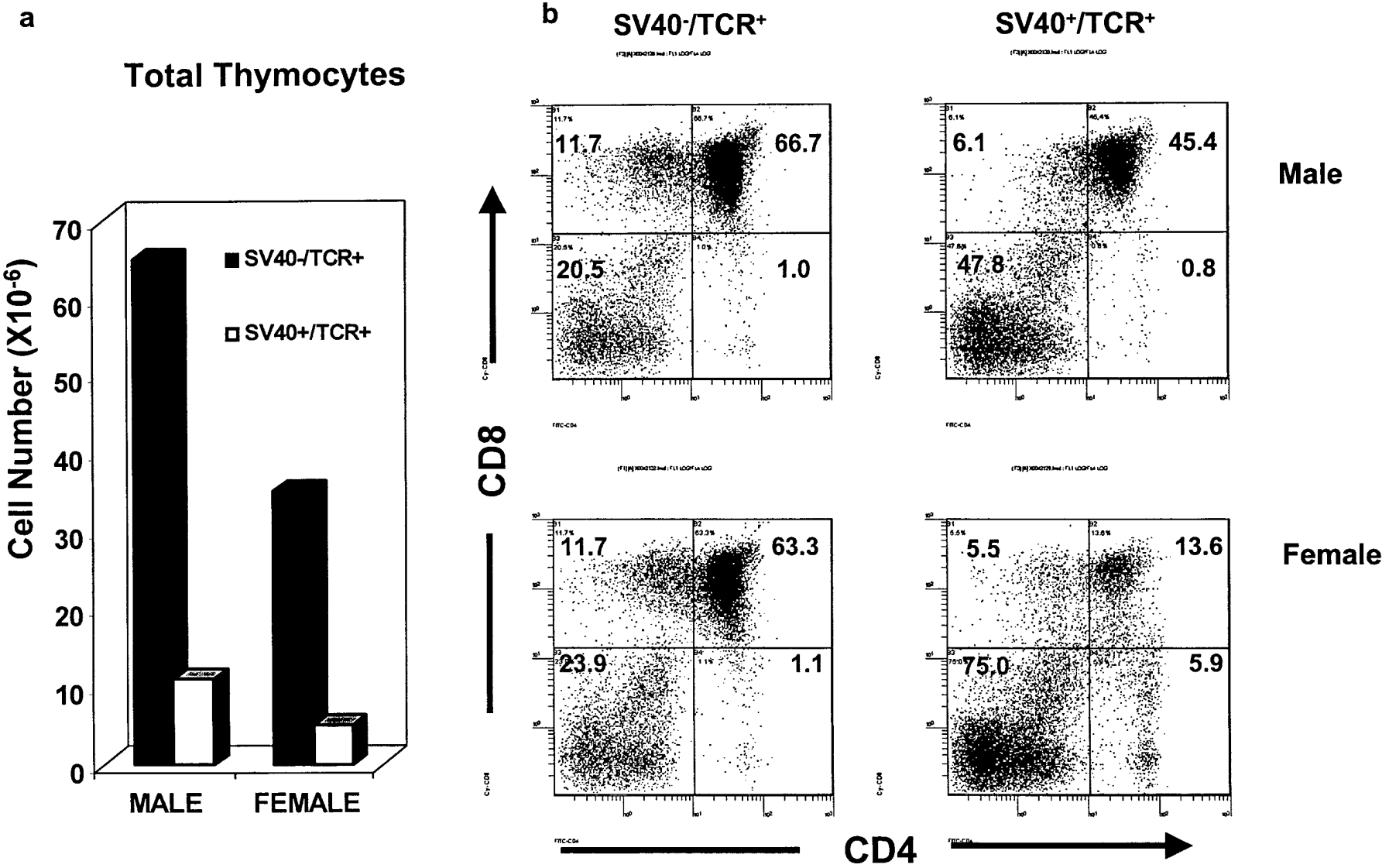

a
Total Thymocytes
Cell Number (X10-6)
70
60
50
40
30
20
10
0
SV40-/TCR+
SV40+/TCR+
MALE
FEMALE
b
SV40-/TCR+
SV40+/TCR+
11.7
66.7
20.5
1.0
6.1
45.4
47.8
0.8
Male
11.7
63.3
23.9
1.1
5.5
13.6
75.0
5.9
Female
CD8
CD4

Fig.2

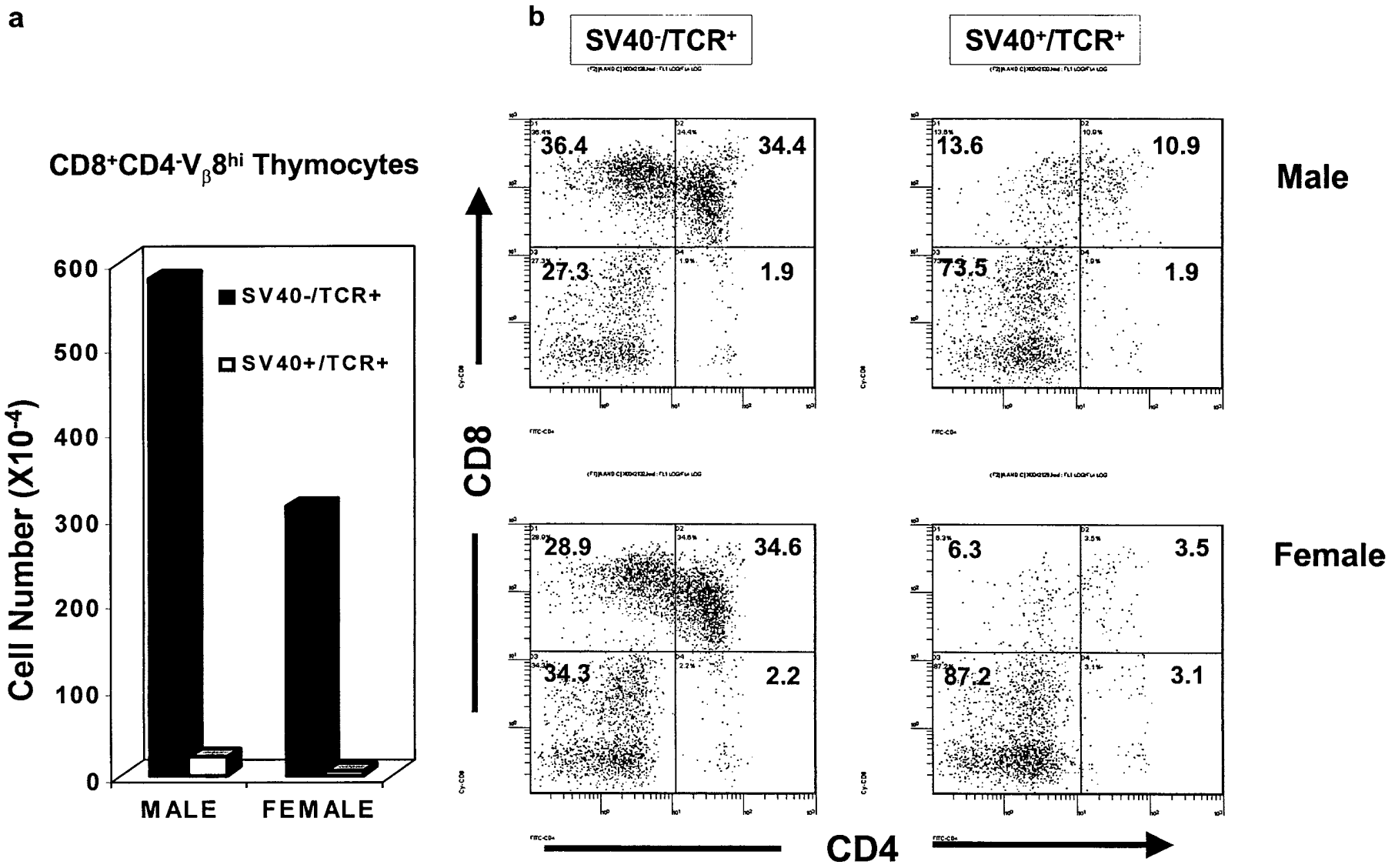

a
CD8+CD4-Vβ8hi Thymocytes
Cell Number (X10-4)
600
500
400
300
200
100
0
SV40-/TCR+
SV40+/TCR+
MALE
FEMALE
b
SV40-/TCR+
SV40+/TCR+
36.4
34.4
27.3
1.9
13.6
10.9
73.5
1.9
28.9
34.6
34.3
2.2
6.3
3.5
87.2
3.1
Male
Female
CD8
CD4

Fig.3

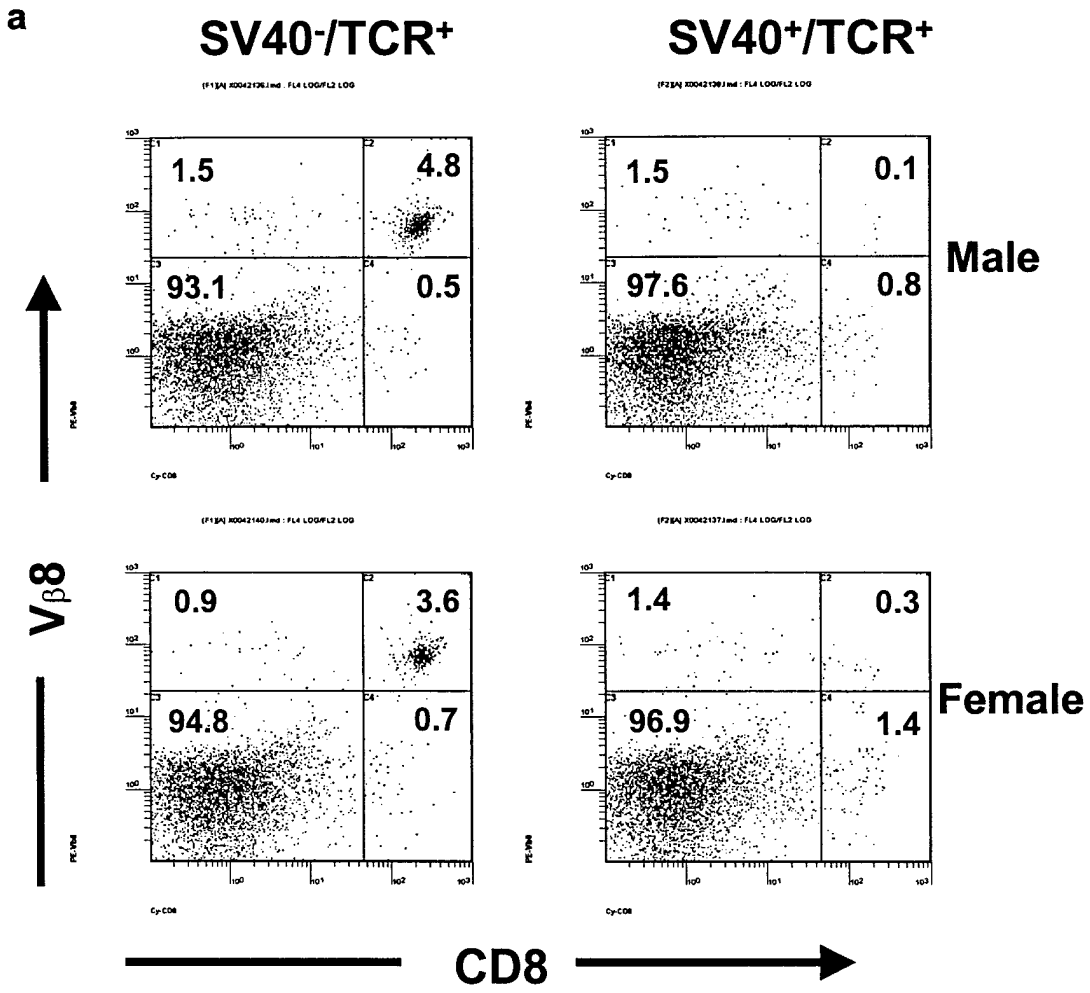

a
SV40-/TCR+
SV40+/TCR+
1.5
4.8
93.1
0.5
1.5
0.1
97.6
0.8
Male
0.9
3.6
94.8
0.7
1.4
0.3
96.9
1.4
Female
Vβ8
CD8

Fig.4a

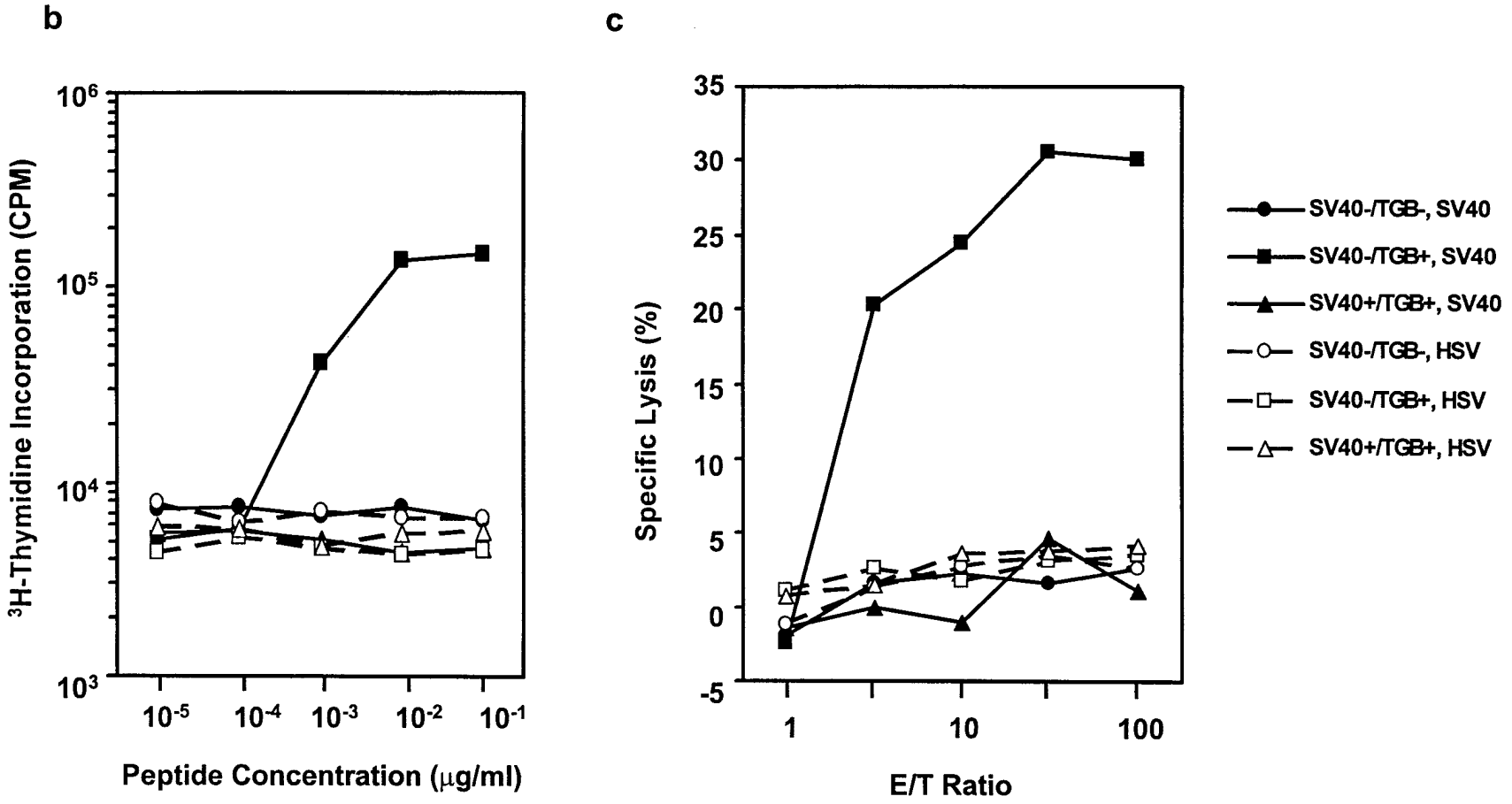

b
^{3}H-Thymidine Incorporation (CPM)
10^6
10^5
10^4
10^3
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
Peptide Concentration (μg/ml)
c
Specific Lysis (%)
35
30
25
20
15
10
5
0
-5
1
10
100
E/T Ratio
SV40-/TGB-, SV40
SV40-/TGB+, SV40
SV40+/TGB+, SV40
SV40-/TGB-, HSV
SV40-/TGB+, HSV
SV40+/TGB+, HSV

Fig.4b

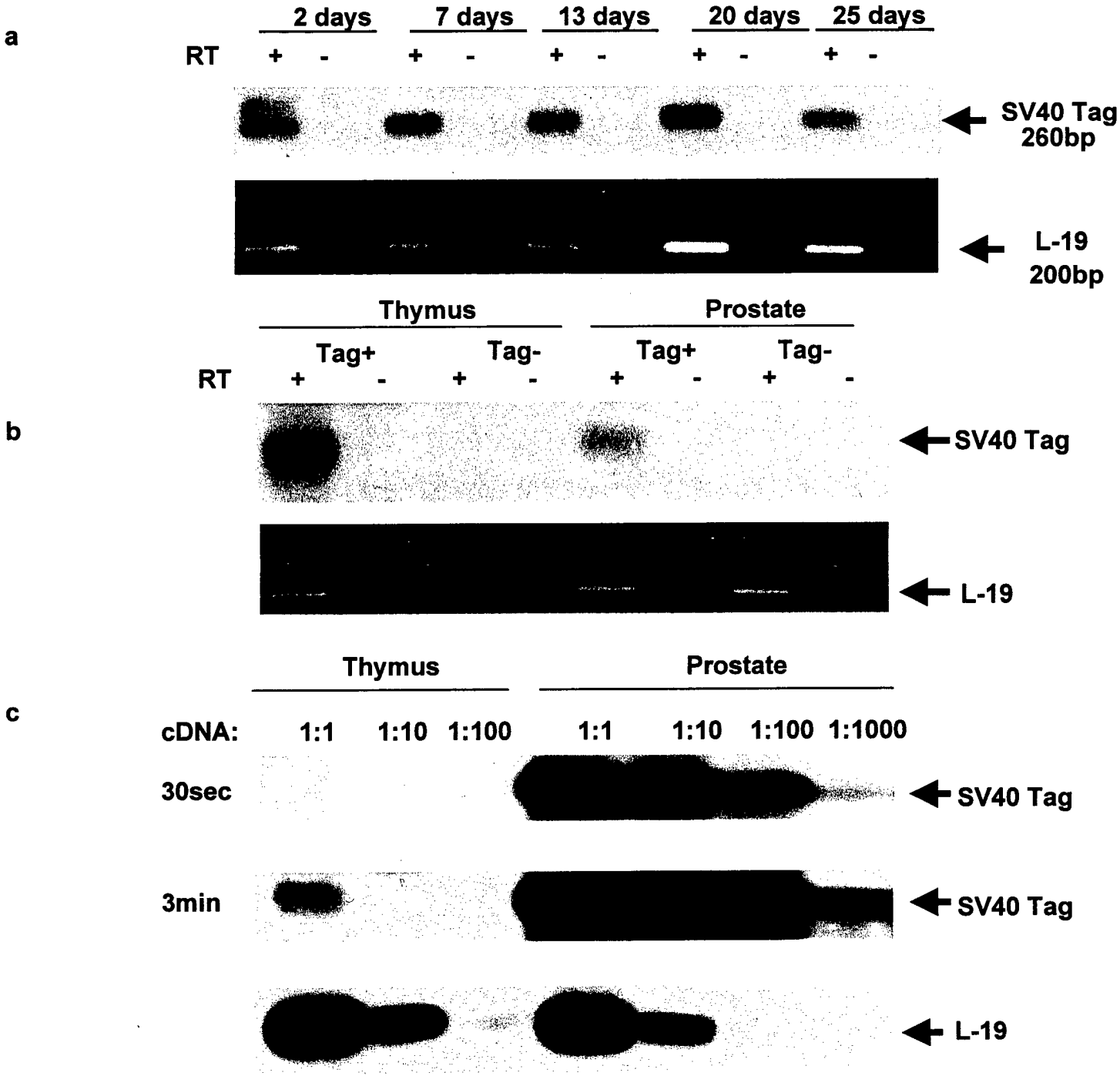
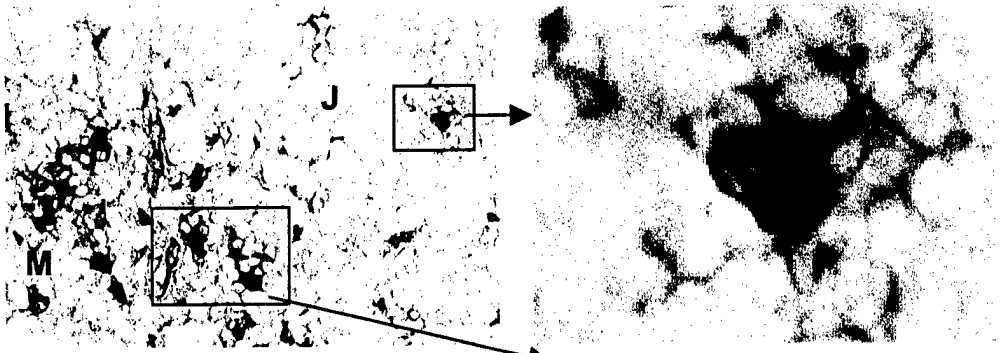

a
2 days
7 days
13 days
20 days
25 days
RT
+ - + - + - + - + -
SV40 Tag
260bp
L-19
200bp
b
Thymus
Prostate
Tag+
Tag-
Tag+
Tag-
RT
+ - + - + - + -
SV40 Tag
L-19
c
Thymus
Prostate
cDNA:
1:1 1:10 1:100 1:1 1:10 1:100 1:1000
30sec
SV40 Tag
3min
SV40 Tag
L-19

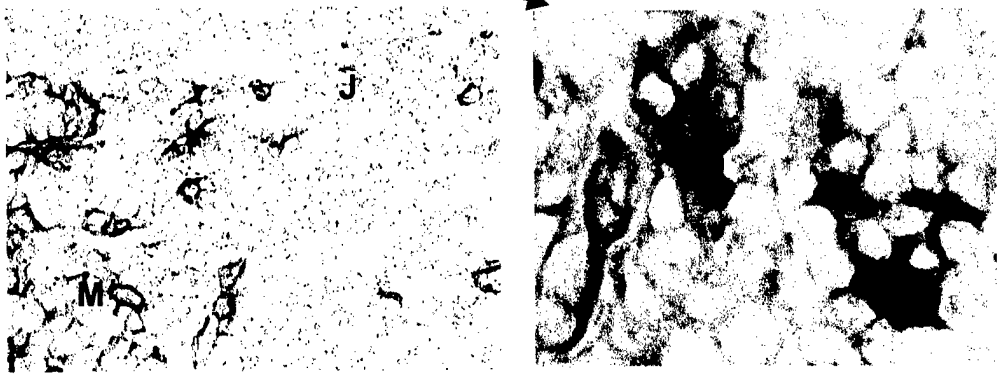
Fig.5

Anti-SV40 Tag
Anti-CD11c
(200X)



Anti-SV40 Tag
Anti-CD11c
(400X)

Anti-CD11c
(200X)



Anti-SV40 Tag
Anti-CD11c
(400X)
Medullar DC

Fig.6

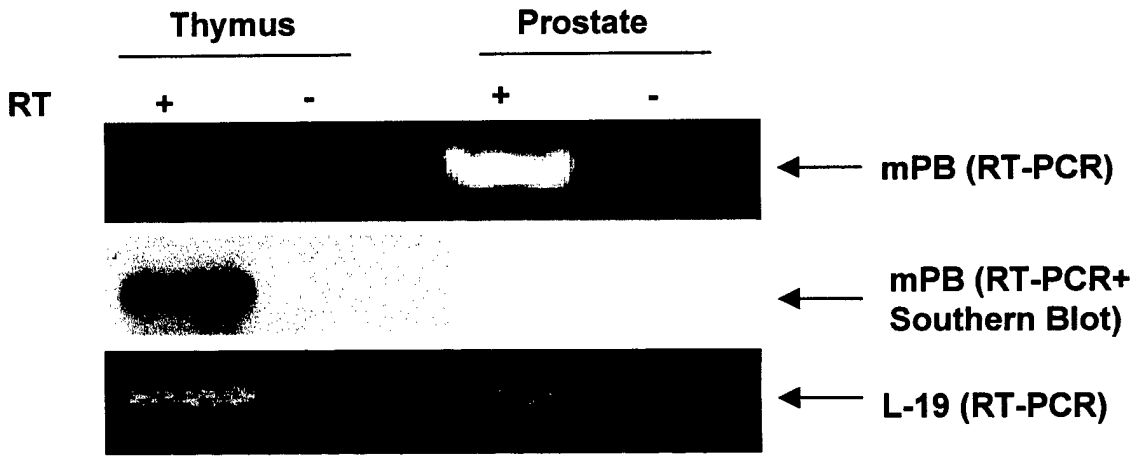

Thymus
Prostate
RT
+
-
+
-
mPB (RT-PCR)
mPB (RT-PCR+
Southern Blot)
L-19 (RT-PCR)

Fig.7